Apoptosis Modulation in Lymphocytes as Novel Concept for Tolerance Induction and Bone Marrow Transplantation to Alleviate a Renal Tubulopathy

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Contents

Summary

Bone marrow (BM) transplantation is well established in the context of malignancies, however, the risks associated with this procedure mostly hampers widespread clinical application in non-malignant conditions. This is an unfortunate situation, as many experimental studies have shown considerable potential of this treatment in a wide array of diseases. Thus, efforts in developing reduced-intensity conditioning of BM recipients are needed in order to translate the many promising experimental findings to the clinics in the future. During my PhD project, I worked on diverse aspects of BM transplantation in non-malignant conditions. One part of the project is settled in the field of immunology. In organ transplantation, the induction of tolerance towards donor tissues by transplanting BM is a promising strategy to overcome the side effects of life-long immunosuppression. I evaluated the prospects of targeting specific lymphocyte populations with a pro-apoptotic drug, and the implications of such targeting for allogenic BM transplantation. Furthermore, I embarked on studying the engraftment process of syngeneic hematopoietic stem cells upon low-intensity conditioning with this anti-apoptotic drug. In the second part of my PhD project, I evaluated the potential of BM transplantation in improving an inborn kidney disease caused by an intracellular transporter in proximal epithelial cells.

Section 1 gives a general introduction to apoptosis regulation in the immune system. Furthermore, an overview about mechanisms and barriers involved in immunological tolerance and hematopoietic stem cell transplantation is given. Finally, Dent's disease, the tubulopathy we tried to alleviate with BM transplantation, is introduced. **Section 2** comprises the results of the experimental studies. We showed that memory T cells can be successfully targeted with a pro-apoptotic drug, allowing to overcome an important barrier to tolerance induction. Additionally, we found that regulatory T cells, crucial players in mediating immunological tolerance, are resistant to Bcl-2/Bcl-xL inhibition. Thus, we developed a novel concept for tipping the balance between effector and regulatory T cells directly in vivo, resulting in a favourable tolerogenic environment. Furthermore, we showed an expansion of hematopoietic stem cells in the BM upon treatment with a pro-apoptotic drug, and a strong synergism with cyclosporine A. However, we are still speculating about the mechanisms that allow stem cell engraftment under myelosuppression-free recipient

conditioning. Finally, we found that transplantation of healthy BM into mice with Dent's disease significantly improved all parameters of Fanconi syndrome. The detailed mechanisms are still under investigation, but we have shown that the diseased kidneys recruited increasingly healthy BM-derived cells that closely associated with tubuli, and thereby recovered the expression of megalin in the brush border membrane of proximal tubuli cells. **Section 3** summarizes the findings of this PhD project and integrates them in a greater context.

Zusammenfassung

Im Kontext von malignen Tumoren ist die Knochenmark (KM) Transplantation gut etabliert. Allerdings verhindern die mit dieser Prozedur verbundenen Risiken eine weit verbreitete klinische Anwendung bei nicht-malignen Krankheiten. Dies ist bedauernswert, da bisher viele experimentelle Studien ein erhebliches Potenzial der KM-Transplantation zur Behandlung zahlreicher Krankheiten gezeigt haben. Aus diesem Grund wäre es wichtig, in die Entwicklung von neuen, weniger toxischen Protokollen für die KM-Transplantation zu investieren, denn es würde erlauben, die vielversprechenden experimentellen Ansätze eventuell in Zukunft in Patienten umzusetzen. Während meiner Doktorarbeit habe ich mich mit verschiedenen Aspekten der KM-Transplantation für nicht-maligne Indikationen befasst. Ein grosser Teil des PhDs ist auf dem Gebiet der Immunologie angesiedelt. In der Organtransplantation kann Toleranz gegenüber dem Spendergewebe durch die Transplantation von KM vom selben Spender erreicht werden. Dies ist ein vielversprechender Ansatz, da so die Nebenwirkungen einhergehend mit einer lebenslangen Immunsuppression verhindert werden können. In meinen Projekten versuchte ich spezifische Lymphozyten Populationen mit einem pro-apoptotischen Medikament zu eliminieren, und untersuchte anschliessend die Auswirkungen dieser Therapie auf die allogene KM-Transplantation. Außerdem versuchte ich den Einnistungsprozess von syngenen hämatopoetischen Stammzellen im KM zu verstehen. Im zweiten Teil meiner Doktorarbeit untersuchte ich die Anwendung der KM-Transplantation zur Korrektur des Phänotyps einer angeborenen Nierenerkrankung, welche durch einen defekten intrazellulären Transporter verursacht wird.

Kapitel 1 gibt eine Einführung in die Apoptose-Regulation im Allgemeinen und in grösserem Detail im Immunsystem. Zudem gebe ich einen Überblick über die Mechanismen und Hindernisse der immunologischen Toleranzinduktion und eine Einführung ins Thema der hämatopoietischen Stammzelltransplantation. Zum Ende wird Dent's Disease vorgestellt, jene Tubulopathie, die wir mit KM-Transplantation versuchten zu mildern. **Kapitel 2** enthält die Ergebnisse der experimentellen Arbeiten. Wir haben gezeigt, dass Gedächtniszellen erfolgreich mit einem pro-apoptotischen Medikament eliminiert werden können. Zusätzlich fanden wir heraus, dass regulatorische T-Zellen welche für die Immuntoleranz von Bedeutung sind, resistent gegenüber der Bcl-2/Bcl-xL Blockade sind.

Wir etablierten somit ein neues Konzept um das Immungleichgweicht zwischen Effektorzellen und regulatorischen Zellen zu verschieben. Weiter zeigten wir eine Expansion von hämatopoietischen Stammzellen im Knochenmark bei der Behandlung mit einem pro-apoptotischen Medikament, ein Effekt der in Kombination mit Cyclosporin A noch verstärkt wurde. Jedoch können wir über die Mechanismen, welche Stammzelltransplantation unter myelosuppressionsfreien Bedingungen ermöglichen, zum jetzigen Zeitpunkt nur spekulieren. Im letzten Projekt schliesslich fanden wir, dass Dent's Disese durch die Transplantation von gesundem KM deutlich gemildert wird. Auch hier sind die genauen Mechanismen der Verbesserung der Krankheit noch Gegenstand laufender Experimente. Wir haben jedoch schon gezeigt, dass die kranken Nieren vermehrt gesunde KM Zellen rekrutiert haben, welche sich dann in der Nähe der Tubuli niedergelassen haben. Dies führte interessanterweise zur erneuten Expression von Megalin im Bürstensaum der proximalen Tubulizellen. **Kapitel 3** fasst die Ergebnisse dieser Doktorarbeit zusammen und diskutiert sie in einem grösseren Kontext.

Abbreviations

APC .....antigen presenting cell
ATG .....anti-thymocyte globulin
Bcl-2 .....B cell lymphoma 2
BH3 .....Bcl-2 homology 3
BM .....bone marrow
CC16 .....clara cell protein
CD .....cluster of differentiation
CLP .....common lymphoid progenitor
CMP .....common myeloid progenitor
CsA .....cyclosporine A
CTLA-4.....cytotoxic T lymphocyte antigen 4
DC .....dendritic cell
DISC .....death-inducing signalling complex
DN .....double negative
DMSO .....dimethyl sulfoxide
DP .....double positive
DST .....donor-specific transfusion
DT .....diphtheria toxin
EV .....extracellular vesicles
FACS .....fluorescence-activated cell sorting
FADD .....Fas-associated death domain protein
FoxP3 .....forkhead box P3
GITR .....glucocorticoid-induced TNF receptor family-related gene
GMP .....granulocyte-macrophage progenitor
GVHD .....graft-versus-host disease
HLA .....human leukocyte antigen
HSC .....hematopoietic stem cell

IDO .....indoleamine 2,3-dioxygenase
I/R injury.....ischemia/reperfusion injury
KO .....knock-out
LMW .....low-molecular weight
LSD .....lysosomal storage disease
LSK .....lin- c-kit+ sca-1+ cells
LT-HSC.....long-term HSC
mAB.....monoclonal antibody
MEP.....myeloid-erythroid progenitor
MHC .....major histocompatibility complex
MLR.....mixed lymphocyte reaction
MOM .....mitochondrial outer membrane
MPP.....multipotent progenitor
MST .....median survival time
mTEC .....medullary thymic epithelial cells
NK .....natural killer cell
PI .....propidium iodide
PT.....proximal tubule
SP .....single positive
SLE.....systemic lupus erythematosus
ST-HSC.....short-term HSC
TBI.....total body irradiation
Teff .....effector T cell
TCR.....T cell receptor
TNF(-R).....tumour necrosis factor (receptor)
TLI .....total lymphoid irradiation
Tm .....memory T cell
TPO .....thrombopoietin
Treg .....regulatory T cell
WT .....wild-type

1 Introduction

1.1 Apoptosis

The general importance of apoptosis

For multicellular organisms, cell death is a mechanism as essential as cell proliferation, and for homeostasis, these two processes must be tightly balanced. A genetically programmed sequence of molecular events has evolved in order to eliminate superfluous or damaged cells in a well-controlled manner, a process that is called apoptosis. Apoptotic cells have been first recognised by their morphologic features [1]:(1) cell shrinkage with condensation of the cytoplasm and the chromatin, followed by a collapse of the cytoskeleton and chromatin fragmentation, (2) extensive membrane blebbing and separation of cell fragments into apoptotic bodies (budding), and finally (3) the rapid phagocytosis of the apoptotic bodies by neighbouring cells or macrophages [2]. In contrast to necrosis, apoptosis does not cause any damaging inflammatory response.

In normal physiology, apoptosis is crucial in many aspects. During development, apoptosis is for example involved in morphogenesis by removing superfluous cells during limb formation or by hollowing ducts. In the nervous system, neurons are overproduced and only cells that succeed in establishing functional synapses escape apoptosis [3]. In adults, programmed cell death is involved in physiological processes such as bone remodelling, wound healing or involution of the mammary glands, but also in protective processes such as removing mutated, damaged or infected cells [4, 5]. Excessive or insufficient apoptosis is a feature of many human diseases, for example cancer, AIDS, neuro-degenerative diseases such as Alzheimer's disease or Parkinson's disease, just to name a few [2].

The extrinsic and intrinsic apoptosis pathways

The decision of a cell to undergo apoptosis is based on the integration of a multitude of cell intrinsic and cell extrinsic stimuli, which all culminate in the activation of caspases that finally demolish the cell. Caspases are endoproteases that use cysteine as their nucleophilic group to hydrolyse protein bonds, and they form a complex proteolytic system similar to the complement or clotting system. In healthy cells, caspases are present as inactive proenzymes (zymogens called procaspases) that require dimerization and often additionally proteolytic processing for activation [6]. Once active, caspases orchestrate cell death by cleaving major constituents of

the cytoskeleton, the nuclear lamina, the extracellular matrix, as well as by targeting genomic DNA, proteins that are essential in housekeeping functions (e.g. transcription factors) and by fragmentation of organelles [7–10]. All these processes result in the macroscopic changes that are characteristic for apoptotic cells [1]. The final and probably most important step of cell death is the clearance of the cell debris (apoptotic bodies) by phagocytes without releasing any cellular contents to the extracellular milieu [11].

Two main pathways lead to death; the extrinsic (death-receptor) pathway and the intrinsic (mitochondrial) pathway. The extrinsic pathway is triggered by the binding of extracellular death ligands (e.g. FasL, TRAIL or TNF-α) to transmembrane death receptors belonging to the tumor necrosis factor receptor (TNF-R) family [12]. These receptors contain a cytoplasmic death domain, which is essential to activate the apoptotic program. Receptor ligation leads to death receptor oligomerization and recruitment of different adaptor proteins, e.g. Fas-associated death domain protein (FADD). These adaptor proteins provide a link between the death receptors and procaspases (mainly caspase-8), and altogether these elements form a large protein complex called death-inducing signalling complex (DISC). Within these DISCs, procaspases dimerize, resulting in protease activation („induced proximity model“) [13]. These initiator caspases then activate efficiently downstream executioner caspases (mainly caspase-3, -6, -7), leading to amplification of the proteolytic cascade (Figure 1.1). Furthermore, inhibitory molecules are produced to restrain activation of the extrinsic apoptosis pathway and prevent inappropriate cell death. c-FLIP is such a molecule that mimics and dimerizes with caspase-8, and thereby prevents autoactivation of these caspases [14].

The intrinsic apoptosis pathway is activated by many different signals, such as growth-factor deprivation, DNA damage, viral infection, altered redox status, ER stress or developmental cues [15]. Upon activation of this death pathway, pores are formed in the mitochondrial outer membrane (MOM), leading to the release of apoptogenic proteins that are contained in the intermembrane space of mitochondria. The most important molecules are cytochrome C, which is involved in mitochondrial oxidative phosphorylation, and SMAC/DIABLO [16]. Upon mitochondrial release, cytochrome C binds to Apaf1, which leads to a conformational change of this molecule, and oligomerization into wheel-like structures called apoptosome. The apoptosome then recruits ini-

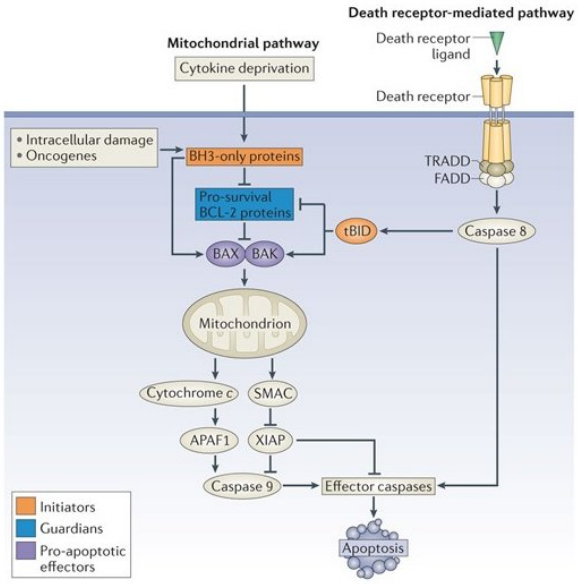

Figure 1.1: The extrinsic apoptosis pathway is activated upon death receptor ligation, leading to recruitment and activation of caspase-8. The Bcl-2 regulated intrinsic apoptosis pathway is activated upon cell intrinsic or extrinsic stimuli such as DNA damage or cytokine deprivation. The two pathways converge at the level of downstream effector caspases, which finally dismantle the cell.
Adapted from Youle et al. [15]

tiator procaspase-9, promotes their dimerization and activation, similar to the activation process of procaspase-8 at the DISC. Caspase-9 further activates the same executioner caspases as caspase-8, thus the final path to death converges in both apoptosis pathways.

As MOM permeabilization is usually a point-of-no-return in cell survival, the processes responsible for pore formation must be very tightly regulated, and members of the Bcl-2 protein family are in charge of this task. These proteins integrate signals from outside or within the cell and set a threshold for apoptosis by interacting with each other at the MOM. Bcl-2 proteins are evolutionary conserved and can be subdivided into three structurally and functionally different subgroups:(1) the pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins, (2) the anti-apoptotic Bcl-2 factors and (3) the pro-apoptotic effector proteins Bak and Bax [17]. The latter two groups share three to four blocks of Bcl-2 sequence homology called BH1-BH4 domains, whereas the BH3-only group, as their name implies, contain only the BH3 domain. BH3 is a amphipathic helix that interacts with a hydrophobic surface groove of the multidomain Bcl-2 members [18, 19].

The interaction between the members of the three subgroups is complex and the proposed models that illustrate the regulation of MOM permeabilization evolved over time. In the beginning, a simple rheostat model

was proposed („ratio between pro- and anti-apoptotic factors"), which later, with the discovery of more pro- and anti-apoptotic factors, was replaced by more complex models. Nowadays, the current consensus is the integration of diverse models called „unified model" (Figure 1.2). Bax and Bak („effectors") are activated by pro-apoptotic BH3-only proteins Bim, Puma and tBID („activators"), which leads to extensive conformational changes and oligomerisation into pore-like structures at the MOM. In healthy cells, anti-apoptotic Bcl-2 family members („guardians": Bcl-2, Bcl-xL, A1, Mcl-1 and Bcl-w) prevent MOM permeabilization by sequestering Bak at the MOM and restraining Bax in the cytosol. The pro-apoptotic „sensitizers" Bad and Noxa cannot directly activate Bax/Bak pore formation, but together with all the other BH3-only proteins, they de-repress Bak/Bax indirectly by binding and inhibiting anti-apoptotic Bcl-2 family members [15–17, 20]. In certain cell types, the intrinsic apoptosis pathway can be activated by caspase-8 mediated cleavage of pro-apoptotic BID, enabling a crosstalk between the extrinsic and intrinsic apoptosis pathway (Figure 1.1) [21].

Enormous complexity is added to the system by the fact that BH3-only factors have binding specificity for certain Bcl-2 homologues, and binding avidity varies among binding partners. Furthermore, the stimuli that activate or promote expression of the diverse factors differ, for example Bim is induced due to growth factor deprivation, while Noxa and Puma are induced by p53 upon DNA damage [17, 22, 23], and post-transcriptional processing is also of importance. The role of individual Bcl-2 factors is often non-redundant, and their relative importance varies greatly in distinct cell populations [24]. And finally, many Bcl-2 factors play also a role in cell physiology outside of apoptosis regulation.

Non-apoptotic cell death

Besides apoptosis, various forms of non-apoptotic cell death exist. Necrosis was long considered to be the counterpart of apoptosis, being an „accidental" or passive form of cell death upon chemical or physical insults, and leading to inflammation and tissue damage [26]. Recent research however has described many modes of „regulated necrosis" that are controlled genetically, similar to apoptosis. All these forms of cell death share morphological features with necrosis [26].

Necroptosis is the best studied mode of such programmed necrosis. It is a well regulated active cell death that occurs under conditions of caspase inhibition [27]. It seems

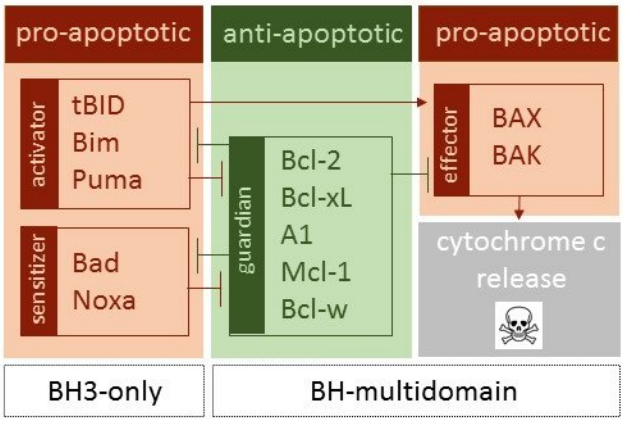

Figure 1.2: Pro-apoptotic BH3-only factors de-repress Bax/Bak-mediated pore formation by blocking anti-apoptotic Bcl-2 factors or by directly activating Bax/Bak. Adapted from Czabotar et al. and Chi et al. [17, 25]

to be a protective mechanism for the host that has co-evolved with viruses, which often encode potent caspase inhibitors. Thus, necroptosis allows virus-infected cells to undergo cell death [28]. It is triggered by death-receptor ligands such as TNF or Fas, and the downstream signalling involves receptor-interacting kinases (RIPK) 1 and 3 [28–30]. However, how necroptosis is eventually executed remains controversial. It has been suggested that mitochondrial dysfunction or plasma membrane permeabilisation are involved [31].

Pyroptosis is an inflammatory programmed cell death that involves the activation of caspase-1 and -11 [32]. Caspase-1 is is mainly known for processing inactive IL-1β, a potent pro-inflammatory cytokine. Activation of pyroptosis results in swelling of the cell, rapid lysis of the plasma membrane and extensive DNA fragmentation [33]. How active caspase-1 kills the cell is completely unknown to date.

Other less well understood modes of programmed necrosis are ferroptosis, oxytosis, parthanatos, just to name a few, and the biological relevance of these processes is unknown [26].

Another mode of non-apoptotic cell death is autophagy; Bax/Bak KO cells that are subjected to cytotoxic stimuli form double-membrane vesicles, that have been shown to be autophagosomes [34]. But again, the role of autophagic cell death under physiological conditions is not well understood yet.

Apoptosis regulation in the immune system

Apoptosis regulation is crucial for the development, maintenance and function of the immune system. Immune cells are overproduced during development, and highly regulated processes select the „right“ cells, allowed or needed in specific contexts, while all unwanted cells are removed. Selection and removal of immune cells is carried out to a major part by dynamic expression of pro-and anti-apoptotic Bcl-2 factors, and the relative importance of these factors changes over the lifetime of immune cells (Figure 1.3). A wide range of developmental cues, such as cytokines or immune receptor ligation, account for this dynamic regulation of Bcl-2 factors. Genetic manipulation of these factors has shown that all cells are fated to die, and their survival depends on one or more anti-apoptotic family members. In contrast, BH3-only proteins mediate cytotoxic or developmental cues, and finally the relative balance between pro-and anti-apoptotic factors determines the fate of a cell [17]. The current knowledge of the importance and function of Bcl-2 factors in physiology and particularly in immune cells, derives to a big part from gain- or loss-of-function studies in mice [24].

Hematopoietic stem cells (HSC) stand at the apex of hematopoiesis and are in charge of continuously replenishing all blood cells. Apoptosis is crucially involved in regulating the size of the HSC pool, as ectopic expression of Bcl-2 caused HSC accumulation, increased in vivo reconstitution potential and enhanced colony forming potential in vitro [35]. However, under physiological conditions, Bcl-2 seems to be less important, as lack of Bcl-2 does not affect early hematopoiesis [36]. In contrast, Mcl-1 is the critical survival factor in HSCs. This factor is highly expressed in HSCs and to a lesser extent in early progenitor populations (common lymphoid and common myeloid progenitors, CLP and CMP). Conditional deletion of Mcl-1 in mixed chimeras with syngeneic labelled wild-type (WT) bone marrow (BM) cells resulted in loss of peripheral cells derived from the Mcl-1 deleted population. Furthermore, mice became very anemic within two to three weeks after complete Mcl-1 deletion due to BM failure [37]. Other cell populations that critically depend on Mcl-1 are natural killer (NK) cells [38], mast cells [39] and plasma cells [40], germinal centre and memory B cells [41]. Mcl-1 is already important in early B-cell development, as Mcl-1 deletion results in a developmental arrest at the pro-B cell stage [42].

Regarding Bcl-xL, the erythroid lineage seems to be most dependent on this survival

factor. Platelets critically depend on Bcl-xL, and loss of this factor results thrombocytopenia [43]. Furthermore, Bcl-xL is needed in erythrocytes at the end of maturation, thus loss of Bcl-xL results in hemolytic anemia [44]. Additionally, reduced plasma cell numbers have been reported in knock-out (KO) animals [41].

Bcl-2 plays an important role in the lymphoid system, as Bcl-2 KO mice loose their mature lymphocyte population [36, 45], but otherwise it has an surprisingly restricted role in normal physiology [24].

Mice have an A1 gene locus quadruplication, encoding for three functional A1 proteins and one pseudo-gene. This hindered the generation of genetic KO models so far, and thus the role of A1 in steady state of immune cell populations is still elusive [46].

Apoptosis modulation during the life of a T cell

T cells undergo a highly orchestrated and complex development and maturation process, and programmed cell death plays a central role in it. Thymocytes have to pass several critical checkpoints to guarantee a functional T cell receptor (TCR) repertoire in the absence of non-functional or autoreactive TCRs. It is assumed that about 95% of thymocytes undergo apoptosis during development.

Immature T cell precursors migrate from the BM to the thymus. These early T cells are in the double negative (DN) development stage and do not express a TCR nor CD4 or CD8 co-receptors. The survival of these cells depends on IL-7 signalling, which directly induces anti-apoptotic Mcl-1 and Bcl-2 expression in early DN stages, that then counteract Bim mediated apoptosis [47–49]. Later, DN thymocytes rearrange their TCRβ locus and present a pre-TCR at their surface. Successful signalling through the pre-TCR results in upregulation of A1, sustained Mcl-1 expression and protection from apoptosis [50, 51], while death receptor signalling deletes cells that fail to present a functional pre-TCR [52].

At the double positive (DP) stage, thymocytes concurrently express CD4 and CD8 co-receptors and rearrange their TCRα chain, which is followed by rigorous TCR testing steps. Only a few thymocytes successfully generate a functional TCR and pass positive selection by recognizing MHC:self-peptide complexes presented by thymic epithelial cells, while the vast majority of thymocytes activate the intrinsic death pathway and undergo apoptosis by neglect. Spontaneous thymocyte apoptosis involves the pro-apoptotic action of Bim and Bax/Bak [53,

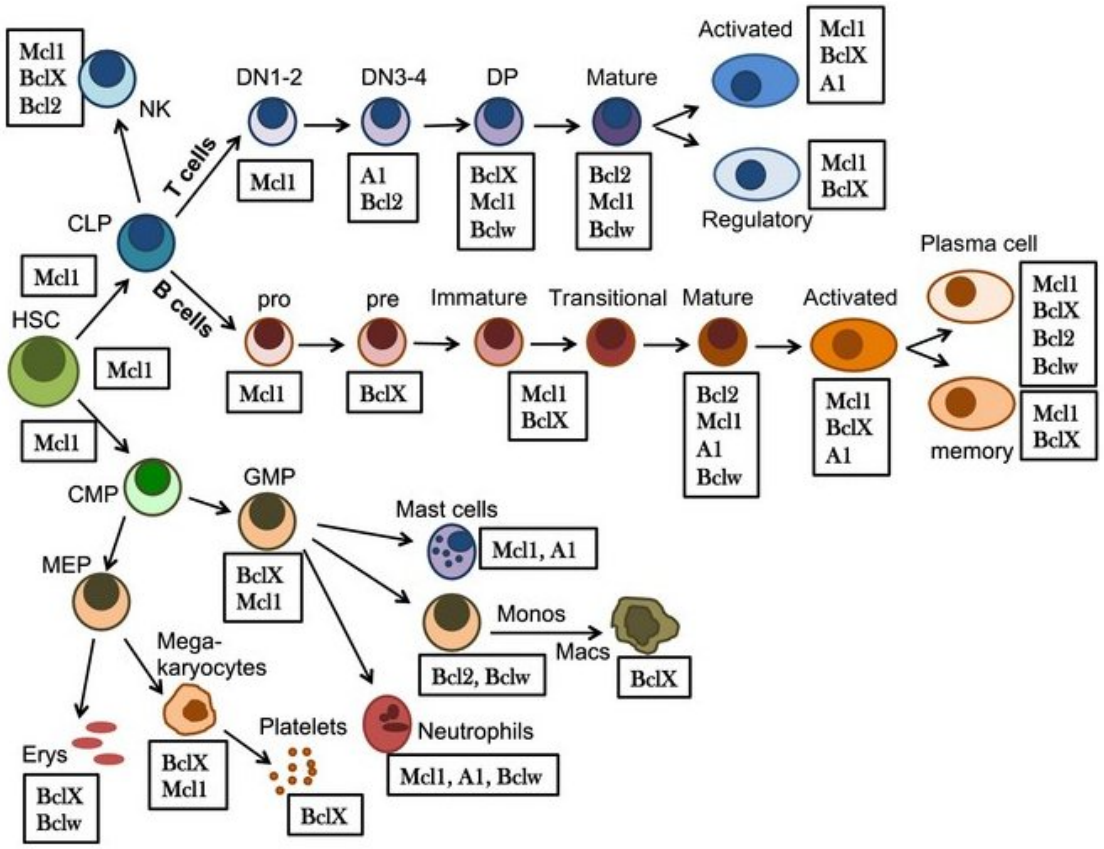

Figure 1.3: Dynamic change of the importance of Bcl-2 factors during hematopoiesis and immune cell differentiation.
Adapted from Sochalska et al. [24]

54], while survival is mediated by high levels of Bcl-xL expressed in DP thymocytes [55].

Following successful positive selection, DP thymocytes downregulate either their CD4 or CD8 receptor and undergo as single positive (SP) cells further maturation steps. Cells that express a TCR with high affinity to MHC:self-peptide complexes are deleted from the T cell repertoire in order to minimize the risk of self-reactivity in the periphery [56]. Bim is critical for this deletion process, as it is upregulated upon high-affinity TCR ligation and thus neutralizes anti-apoptotic Bcl-xL [57]. This process, which is the most important checkpoint for central tolerance, is called negative selection. Taken together, apoptosis is a fundamental process involved in the development of

a functional T cell repertoire in the thymus.

Mature CD4 or CD8 T cells that survive the stringent selection processes in the thymus migrate to the periphery. Homeostasis and maintenance of the naïve T cell pool requires IL-7 and TCR-MHC signalling, and Bcl-2 is considered to be the most important effector downstream of these signals [58–60]. But also expression of Mcl-1 is essential for the survival of mature T cells, as specific deletion of Mcl-1 in lymphocytes results in severe lymphopenia [47]. In contrast, Bcl-xL deletion leads only to a minor reduction of mature T cells [51]. Upon cognate antigen encounter, CD8 T cells become activated and undergo vigorous proliferation and differentiation into cytotoxic effector T cells (Teff). Following TCR stimulation, anti-apoptotic A1 and Bcl-xL are upregulated, while an increased Bim level primes cells to death [61, 62].

In order to prevent immunopathology, the effector T cell pool contracts after the peak of the immune response, and 90-95% of Teffs are eliminated. Activation-induced cell death by Fas ligand has been proposed to be one of the mechanisms controlling effector pool contraction; however, in vivo contraction is not affected in animals that lack death receptor signalling [63–65]. On the contrary, the intrinsic apoptosis pathway is activated as cytokines become a limiting factor towards the end of an immune response. Cells enter a pro-apoptotic state that is mediated by:(1) already high Bim levels as a result of TCR stimulation, (2) cytokine-deprivation mediated Puma upregulation and (3) concomitant Bcl-2 and Bcl-xL cleavage [65–68]. Only few cells survive the contraction phase and differentiate into long-lived memory cells (Tm). The balance between IL-7 and IL-15 driven Bcl-2 expression and Bim is responsible for maintenance of Tms [60, 69]. Differential apoptosis regulation among different T cell subsets (e.g. the various T helper cell subsets) has not been investigated in detail to date. However, a recent publication identified Mcl-1 as the predominant survival factor in regulatory T cells (Tregs). Mcl-1 can be considered as rheostat for controlling the Treg homeostatic niche, and it is positively regulated by a IL-2 feedback loop [70]. Treg-specific deletion of Mcl-1 led to rapid onset of fatal autoimmunity, and Bim was identified as the main antagonist of Mcl-1 in Tregs [70]. Although this publication states that Bcl-xL is dispensable for Treg survival, an other publication showed increased Bcl-xL mRNA expression levels in CD4+FoxP3+ cells in comparison to CD4+FoxP3- cells [71].

Diseases caused by defective apoptosis regulation in lymphocytes

As apoptosis is fundamental to such a wide extent in taking life-versus-death decisions in lymphocytes, one can well conceive the severe consequences of dysregulated or defective apoptosis. Indeed, apoptosis defects are at the basis of many immunological diseases and hematological malignancies. Mouse knock-out or knock-in models have provided insights into disease mechanisms.

Apoptosis seems to play a pivotal role in the pathogenesis of autoimmunity. As explained above, on one hand, functional apoptosis results in the clearance of cell debris of dying cells without inducing inflammation in surrounding tissues. On the other hand, both apoptotic pathways are responsible for establishing central tolerance and maintaining peripheral tolerance. Defects in both processes can result in breakdown of tolerance to self-antigens. *Lpr* and *gld* mice lack Fas or FasL, respectively. These mice develop massive lymphadenopathy and splenomegaly associated with proliferation of aberrant T cells, and B cells produce large amounts of autoantibodies that cause arthritis and immune complex glomerulonephrosis [72–74]. Human autoimmune lymphoproliferative disease (ALPS) involves mutations in the same genes, while mice also present phenotypic but non-orthologous similarities with human systemic lupus erythematosus (SLE) [75]. SLE, Sjorgen's syndrome and systemic sclerosis may all result from defective burial phases of apoptosis, which might be a continuous source of autoantigens that trigger lymphocyte activation [76]. The Bcl-2 family forms a barrier against autoimmune disease [77]. The lack of Bim or overexpression of Bcl-2 in mice leads to an SLE-like disease due to perturbed B and T cell development and homeostasis [53, 78]. So far, overexpression of various anti-apoptotic factors have been reported in SLE patients, and this overexpression was often mediated by a dysregulated cytokine milieu [79–82]. No loss-of-function mutations in autoimmune diseases have been reported, however, accelerated degradation and thus decreased Bim levels led to ALPS-like disease in one patient [83].

Another area where dysregulated apoptosis can have devastating consequences is cancer, as evasion of apoptosis is one of the hallmarks of cancer [84]. Transgenic overexpression of Bcl-2 in T and B cells results in increased cell numbers and higher incidence of malignant transformation in mice [85, 86]. Accordingly, the t(14;18) translocation, which results in Bcl-2 overexpression, is the most common translocation in human

lymphoid malignancies and defines Bcl-2 as oncogene [87].

Pharmacological modulation of the intrinsic apoptosis pathway

Specific targeting of overexpressed anti-apoptotic Bcl-2 family members in cancers bears great therapeutic potential, as it can overcome drug resistance to chemo- and radiotherapy. The first strategy to target Bcl-2 was its downregulation by an antisense oligonucleotide. A compound called oblimersen sodium entered clinical trials, but its success was limited [88]. Much more successful was the strategy to target the BH3-binding groove present on anti-apoptotic Bcl-2 proteins with small molecule inhibitors termed „BH3 mimetics“. If not blocked, the hydrophobic BH3-binding groove („receptor“) interacts with the conserved BH3 domain („ligand“) present on all pro-apoptotic proteins, and thereby prevents MOM pore formation and apoptosis [89].

So far, more than 20 BH3 mimetics with different potencies and selectivities have been developed and tested in vitro and in pre-clinical cancer models, and some have entered (more or less successful) clinical trials. Figure 1.4 shows some of these compounds and their targets. Maritoclax, MIM1 and the recently discovered A-1210477 are specific and potent Mcl-1 inhibitors [91, 92], ABT-199 is very specific in targeting Bcl-2 [93], and A-1155463 is a Bcl-xL inhibitor [94]. An A1 inhibitor has not been developed yet. Obatoclax (GX15-070), gossypol (AT-101) and its derivatives (e.g. sabutoclax) are pan-Bcl-2 inhibitors. While obatoclax shows off-target toxicity (killing of Bax/Bak KO cells), sabutoclax seems to be much more specific [95, 96].

ABT-737 and its oral analogue ABT-263 (navitoclax) are the best characterized BH3 mimetics so far. ABT-263 is used in clinical studies, while for pre-clinical research the company AbbVie usually only provides ABT-737. This compound selectively binds Bcl-2, Bcl-xL and Bcl-w with a very high affinity (Ki below 1nM) and Mcl-1 and A1 with a much lower affinity [97]. In contrast to some other BH3 mimetics, ABT-737 kills cells only via the intrinsic apoptosis pathway and shows no off-target toxicity [96]. In clinical trials, ABT-263 was safe and generally well tolerated. However, a dose-limiting side effect was reversible thrombocytopenia caused by the Bcl-xL dependence of platelets [98].

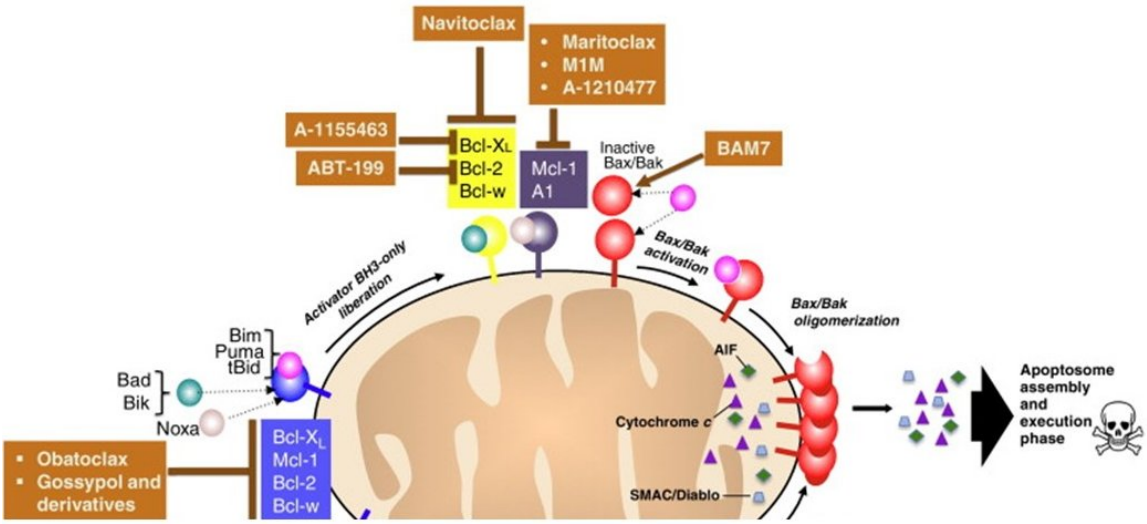

Figure 1.4: BH3 mimetics and their targets: All these compounds have been reported to induce apoptosis in preclinical cancer models or clinical trials.
Adapted from Vela and Marzo [90].

1.2 Mechanisms and barriers involved in immunological tolerance

The immune system has evolved to protect us by mounting effective immune responses against foreign pathogens, while at the same time showing no reactivity against our own tissues. This fundamental paradigm is called „immune tolerance“. Immune tolerance is achieved through the complementary action of deletion and regulation, encompassing both, central and peripheral mechanisms (Figure 1.6) [99]. In the context of organ transplantation, the initial definition of tolerance has been expanded. Here, the ultimate goal is to extend the immune-self and render the immune system specifically unresponsive towards the allograft [100].

Several non-redundant mechanisms ensure immunological tolerance. The most important one is „central tolerance“. Random recombination of the α- and β-chain generates a wide diversity of TCRs, of which a large part is self-reactive. Thus as outlined earlier, T cells must undergo a stringent selection process in the thymus in order to assure a self-recognising and -tolerant T cell pool. DP thymocytes that recognize MHC:self-peptide complexes are positively selected and become SP cells. During negative selection (also known as clonal deletion), self-reactive T cell precursors are

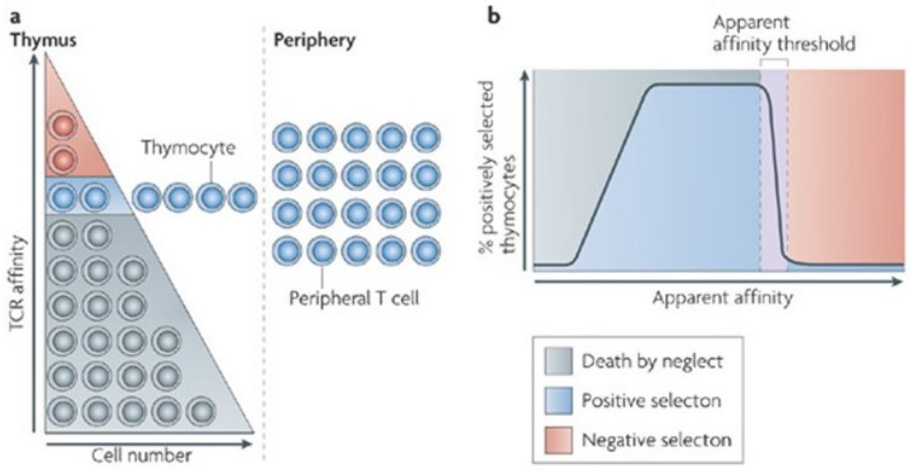

Figure 1.5: The peripheral T cell pool is generated through the selection of self-restricted (positive selection) and self-tolerant (negative selection) T cell progenitors in the thymus. Most developing cells possess TCRs that don't recognize MHC:self-peptide complexes, thus they don't receive survival signals and die by neglect (grey). High affinity binding to MHC:self-peptide complexes induces apoptosis (negative selection, red). Thymocytes that bind MHC:self-peptide complexes with low affinity are positively selected (blue) and develop into mature T cells.
Adapted from Palmer and Naeher [101].

deleted, and for this process the transcription factor Aire is crucial. Aire is selectively expressed in medullary thymic epithelial cells (mTEC) and drives the ectopic expression of tissue-specific antigens. Recognition of these antigens results in deletion of developing thymocytes [102]. Taken together, weak TCR signalling protects thymocytes from death by neglect, while high-affinity interactions delete the cells (Figure 1.5). Slightly higher affinity for MHC:self-peptide complexes are permissive for Treg differentiation, leading to a Treg-specific TCR repertoire that is skewed towards self-reactivity [103].

However, some self-reactive T cells are released into the periphery as the Aire-dependent deletion process is not perfect. Thus, several peripheral immune tolerance mechanisms come into operation. An important basic mechanism is „ignorance", which is the physical separation of self-reactive T cells from parenchymal cells that express tissue antigens [104]. Naïve CCR7+ CD62L+ T cells recirculate between blood, secondary lymphoid organs and the lymph system and thus not necessarily come into contact with peripheral antigens. Anergy (functional inactivation) or deletion occurs as result of TCR-stimulation in the absence of costimulation. Tissue-resident dendritic cells (DCs)

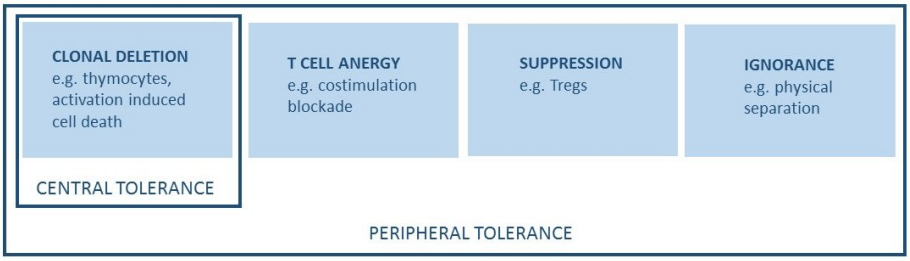

Figure 1.6: There are four major mechanisms for induction and maintenance of T cell tolerance. Clonal deletion, anergy and ignorance are passive tolerance mechanisms and suppression by Tregs is actively maintained tolerance. Clonal deletion is mainly taking place in the thymus (central tolerance) but also in the periphery e.g. after antigen encounter. Anergy, Treg-mediated suppression and ignorance are important for peripheral tolerance.

phagocyte cell debris from apoptotic cells that arise from normal cell turnover and present the antigens to naïve T cells in the draining lymph nodes. In the absence of an infection, DCs are not activated and thus only express low levels of costimulatory molecules, resulting in anergy or apoptosis of T cells [105]. Finally, Treg-mediated active suppression of self-reactive T cells another essential tolerance mechanism (Figure 1.6).

Regulatory T cells

The best studied regulatory cell population in mice are CD4 Tregs that are defined by their constitutive expression of the FoxP3 transcription factor and the high-affinity α-chain IL-2 receptor (CD25) at their surface [106, 107]. Furthermore, expression of several molecules that are needed for the immunosuppressive function are characteristic for Tregs. They represent between 5-10% of the peripheral CD4 T cell population, and most Tregs arise from the thymus and thus are termed „thymus-derived Tregs“ (tTregs). Under certain conditions, Tregs can develop from conventional effector CD4 cells in the periphery („peripherally-derived Tregs“ pTregs) or in vitro (iTregs). The crucial importance of Tregs in peripheral tolerance is demonstrated in patients with the X-linked immunodeficiency syndrome IPEX and in scurfy mice. Both have mutations in the FoxP3 locus and present an autoimmune disease in multiple endocrine organs, inflammatory bowel disease, severe allergy with dermatitis, and fatal infections [108, 109]. But also minor genetic abnormalities that affect Treg differentiation, maintenance or function by direct or indirect means can predispose to (organ-specific) autoimmunity, e.g. polymorphisms in the genes encoding for CTLA-4, IL-2, IL-2Rα or TGF-β [110–113].

Four basic mechanisms have been proposed of how Tregs exert their immunosuppressive function: suppression by inhibitory cytokines, cytolysis, metabolic disruption and modulation of DC maturation or function (Figure 1.7). Three inhibitory cytokines (IL-10, IL-35 and TGF-β) are the key mediators of Treg suppressive function. Despite intensive research, the relative importance of each cytokine under different conditions in not completely understood yet. IL-10 is expressed by effector Tregs that localize to mucosal tissues and thus seems to control inflammation at barrier tissues [114, 115]. IL-35 is a potent cytokine required for maximal regulatory activity of Tregs [116], whereas the importance of TGF-β for suppressive function is still controversial [117]. In diverse experimental settings, Tregs have been shown to express granzyme B, perforin, TRAIL and galectin-1, and loss of either mechanism resulted in reduced suppressive activity of Tregs [118–121]. By constitutively expressing CD25, Tregs have been shown to deprive dividing effector cells from IL-2, which leads to apoptosis of these cells [122]. And finally, Tregs are able to influence effector cell activation indirectly by modulating the maturation and activity of DCs. For example, Treg can induce the expression of the immunoregulatory enzyme 2,3-dioxygenase (IDO) in DCs, which then is secreted and thereby suppresses Teff function [123]. Additionally, CTLA-4 expressed on Tregs can interact with CD80 and CD86 on DCs, and by a process called trans-endocytosis, Tregs thereby remove CD80/86 from the surface of DCs. Hence, DCs cannot provide T cells with costimulation through CD28, which inhibits excessive activation of Teffs [124].

Tolerance induction strategies in transplantation

Transplantation is often the best treatment option for patients with end stage organ failure. However, the transplanted organ primes innate and adaptive immune responses due to tissue injury and the foreign antigens presented on the tissue. With current immunosuppressive treatments, acute rejection can be prevented efficiently [125]. In the long-term however, little progress has been made and chronic rejection is still causing considerable organ loss. Furthermore, the generalized immunosuppression bears an increased risk for infections (including opportunistic infections) and certain cancers [126]. For these reasons, one of the most important goals in the transplantation field is the induction of tolerance, which is immunological unresponsiveness towards the allograft without the need for immunosuppression. The

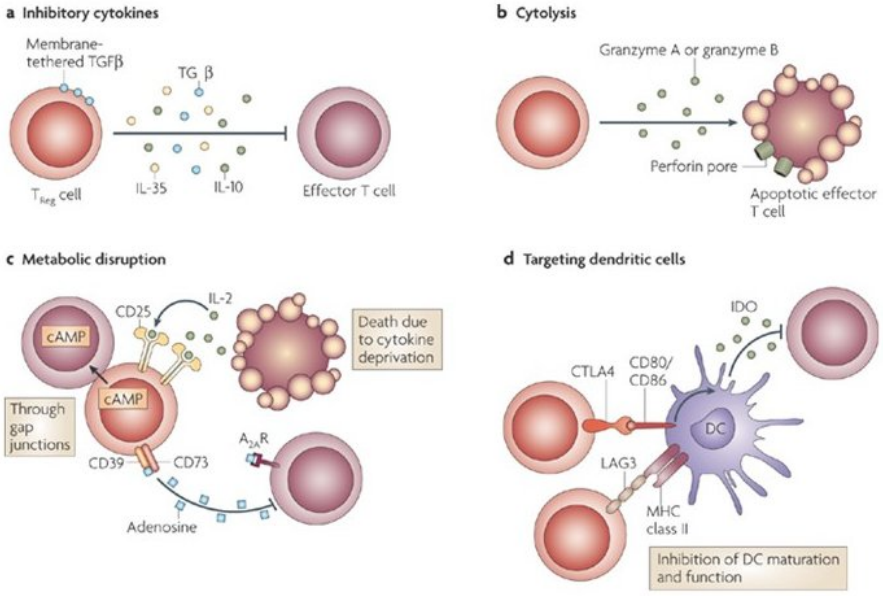

Figure 1.7: Four basic mechanisms of Treg-mediated suppression of effector T cell viability and function. Adapted from Vignali et al. [117]

detailed understanding of the processes that mediate and maintain self-tolerance can be exploited to develop strategies to induce allo-specific tolerance.

Two immunological barriers have to be breached for successful tolerance induction. On one hand, the pre-existing T cell pool in the periphery needs to be tolerized to alloantigens, while on the other hand, newly developing allo-reactive T cells in the thymus need to be eliminated. The power of the peripheral T cell pool is illustrated by the fact that thymectomized rats still can reject skin grafts [127]. Thus, several peripheral tolerance approaches that target mature T cells have been proposed. Early approaches targeted either CD4, or CD4 plus CD8 co-receptors, with depleting or non-depleting antibodies, which allowed prolonged heart and skin graft survival [128, 129]. Later, combined blockade of the CD28 (CTLA-4 Ig) and CD40 (anti-CD154, MR1) costimulatory pathways proofed successful in preventing heart (>80 days) and skin (>50 days) allograft rejection [130]. Another strategy was to combine donor-specific transfusion (DST) with monoclonal antibodies such as non-depleting anti-CD4, MR1 or CTLA-4 Ig [131–133]. Depending on the transplant model and the strain combination used,

graft rejection could be delayed or prevented. With the DST plus MR1 approach, „functional tolerance“ was only achieved in less stringent transplants like heart or islet cells, while long-term skin graft survival required concomitant thymectomy [134]. Similar to physiological tolerance induction, peripheral allo-tolerance is based on deletion/anergy and active regulation [99]. Especially suppression of allo-reactivity by Tregs is an important mechanism in most approaches that aim at inducing peripheral tolerance. In fact, it has been stated that the current paradigm is that „the balance of alloantigen-reactive Treg cells and Teff cells ultimately determines whether the graft is accepted or rejected“ [135]. Thus, direct pharmacological targeting of pathways that promote Treg development or expansion in vivo, or ex vivo generation and/or expansion of Tregs represent other promising strategies to promote graft survival.

Tolerance is a graded condition. For example, tolerance can be „operational “, which means that a graft may be accepted without immunosuppression, but a second graft will be rejected. Although peripheral tolerance strategies allow successful operational tolerance induction, effective long-term and systemic tolerance can only be achieved with central tolerance mechanisms (at least in higher animals and humans). This approach will be discussed in detail in section 1.3.

Memory T cells

Immunological memory is a key feature of adaptive immunity. Upon antigen encounter, naïve T cells proliferate extensively and most of them differentiate into short-lived Teffs that undergo apoptotic cell death after antigen clearance. In contrast, a small subset of the Teff pool differentiates into memory cells by activating a distinct pattern of transcription factors [136], resulting in longevity, a distinct dependency on cytokines (mainly IL-7 and IL-15) and differential migration patterns as compared to naïve T cells [137]. There are three main subsets of CD8 Tm: central and effector memory cells (Tcm and Tem), both recirculate in the blood, and non-circulatory tissue-resident Tm [137, 138]. Upon re-encounter of their cognate antigen, Tms show a rapid and robust immune response with a low activation threshold [139]. Besides their need for lower antigen doses for activation, Tm are also less dependent on „conventional“ costimulatory signals (CD28 and CD154) compared to naïve T cells [140–142]. For these reasons, Tm can be fully activated by non-professional antigen-presenting cells in tissues (e.g. endothelial or parenchymal cells). Once re-activated, Tm acquire an

effector phenotype and thereby exert important functions in protecting the host from reinfection with previously encountered antigens.

While Tm are well equipped for fighting infections and certainly are important for protection, they pose problems in the transplantation field. Individuals with a normal infection and vaccination history are likely to possess alloreactive memory T cells in their T cell repertoire, and these cells are difficult to circumvent by immunosuppression and pose a barrier towards tolerance induction strategies. Several mechanisms lead to the generation of alloreactive Tm in an individual:(1) direct sensitization with alloantigens, (2) antigen-independent Tm generation, or (3) cross-reactivity with another antigen [143]. Direct sensitization can occur upon contact with allogenic antigens, e.g. prior grafts, blood or platelet transfusions or during pregnancy, where the mother can be primed against paternal antigens carried by the foetus [144–146]. Interestingly, cells with Tm-like properties and phenotype can also develop in an antigen-independent manner during homeostatic proliferation after non-specific T cell depletion. Naïve alloreactive T cells proliferate under such lymphopenic conditions in response to IL-7 and differentiate into Tms [147–149]. This finding is important, as lymphocyte depletion is frequently used as induction therapy in sensitized transplant patients.

The most important process of alloreactive Tm generation is termed „heterologous immunity“ [150]. During the course of an infection, virus-specific Tm are generated. Because cross-reactivity is a common phenomenon, many cells of a patient's Tm pool possess TCRs that are cross-reactive to alloantigens [151–154]. Indeed, several studies have shown specific cross-reactivity between pathogen-derived epitopes and allogenic human leukocyte antigens (HLA) [155–157]. More recent studies further showed that recognition of unrelated peptide epitopes by T cell clones is based on molecular mimicry [158–160]. Thus, a large proportion of Tm clones present in a patient possesses cross-reactivity for allo-antigens.

Tm-related problems in transplantation

As Tm require lower antigen doses and less costimulatory signals for re-activation, they are perfectly suited to rapidly respond to a recurrent infection [161]. However, these unique features allow alloreactive Tm to traffic rapidly into grafts, recruit innate immune cells and damage the tissue. Conventional immunosuppressive drugs usually target cel-

lular checkpoints that are required for the activation and clonal expansion of naïve T cells, and thus might be less effective in controlling Tm activation [162]. This notion is supported by the fact that immunosuppressed transplant recipients still receive vaccinations that mediate protection at least partially by Tm [163].

Importantly, Tm prevent tolerance induction in protocols that are based on blockade of the CD40-CD154 and CD28-CD80/86 costimulatory axes. As explained before, costimulation blockade with or without DST were successful in inducing tolerance to various grafts. However, mice that were previously primed with allo-antigens or pathogens remained refractory to these experimental strategies [150, 154, 164]. Heterologous immunity thus is a potent barrier to tolerance induction. It explains the difficulties encountered when protocols that were successful in naïve mice are translated to non-human primates or patients.

1.3 Hematopoietic stem cell transplantation

In 1951, researchers noticed that intravenous injection of BM cells from an adult mouse can protect the recipient from lethal irradiation [165]. Since then, HSCs have been studied extensively, they are the best studied somatic stem cell type and can be considered as the „stem cell prototype“. The concept that all types of blood cells (e.g. erythrocytes, platelets and all the immune cells) derive from a common pool of self-renewing HSCs has long been established [166, 167]. HSCs cannot be identified based on their morphology, but flow cytometry allows to characterize these cells based on their surface marker expression. On a functional level, the gold standard test to proof the identity of a HSC is the „competitive repopulation unit assay“, where one single cell is transplanted into a lethally irradiated animal. This allows testing whether the cell of interest is able to regenerate and maintain long-term blood production [168]. However, recent research questions this approach [169]. Also the notion of „the“ HSC is wrong, because there exist diverse HSC subsets with a considerable heterogeneity in self-renewal and differentiation potential, which is a subject of intensive research and much debate [168]. Some reports claim transdifferentiation of HSC-derived cells into non-hematological tissues, however experts in the field of HSCs question such observations [170].

The BM microenvironment harbours not only rare HSCs and their progeny, but also non-hematopoietic cells belonging to the mesenchymal lineage that support

hematopoiesis. Mesenchymal stromal cells (MSCs) show self-renewal and have a great differentiation potential. They can give rise to osteoblasts, chondrocytes and adipocytes, but true „stemness“ is difficult to prove experimentally [171, 172]. Nevertheless, MSCs have attracted great interest in regenerative medicine. They can be easily isolated from the BM and injected systemically. It is controversial whether MSCs specifically home to tissues, however it has been shown that they are recruited to sites of acute injury, e.g. to the kidney [173]. MSCs secrete a vast diversity of soluble factors, and their regenerative capacities have mainly been attributed to their paracrine/endocrine effects. Reports claim transdifferentiation of MSCs into other tissues such as neurons or muscles, but this is a topic of much debate. What is clear however, is the fact that MSCs have a profound impact on immunity by interacting with almost all immune cells [174].

When we perform experimental BM transplantation in mice, most of the time we isolate all the cells from donor BM and inject the complete mixture (unfractionated BM) intravenously into a pre-conditioned recipient. This cell cocktail consists of a few HSCs, many progenitors of the different blood lineages at various development stages, erythrocytes, platelets and mature immune cells such as T and B lymphocytes. In addition, there are MSCs and their progeny. After systemic injection, HSCs and possibly also early progenitors readily home to the BM of the recipient, while mature lymphocytes persist for quite a long time in the periphery. Preparatory irradiation of the recipient results in damage of MSCs and disruption of the HSC niche, but endogenous MSCs are not killed [175]. Co-injected donor MSCs do not home to the BM, therefore MSCs remain of host origin [176]. But co-transplanted MSCs have an important function in promoting HSC engraftment, supporting reconstitution of hematopoiesis by producing cytokines, and possibly also by preventing graft-versus-host disease (GVHD) [177].

As alternative to using unfractionated BM for transplantation, HSCs can be enriched by depleting all lineage-positive cells from the BM suspension by MACS, or by FACS sorting of highly purified HSCs populations based on surface marker expression. In clinics, mostly peripherally mobilized and purified CD34+ HSCs, sometimes in conjunction with T cells, are transplanted. In an experimental therapeutic/regenerative setting, BM-derived cells might be administered not necessarily with the aim to have permanent engraftment (without preparative conditioning, as explained in detail later in this sec-

tion), but to profit from short-term effects. These positive effects may derive from both populations present in the BM, HSCs and MSCs.

Clinical indications for HSC transplantation

Indications for HSC transplantation are diverse. Hematologic malignancies that are refractory to other treatments are the main indication for HSC transplantation. Here, maximally tolerated myelotoxic doses of chemo- and radiotherapy are needed to eliminate the cancer, and the resulting marrow aplasia is treated with the transplantation of autologous or allogenic HSCs [170].

Additionally, there is wide variety of non-malignant conditions that (potentially) profit from HSC transplantation. Depending on the severity of the condition and other treatment opportunities, more or less toxic preparatory regimen and the associated side effects are acceptable. It can be performed in transplant recipients to deliberately reprogram their immune system and render it tolerant to allo-antigens. This approach will be discussed in the following section. Additionally, there are many diseases of the immune system or the hematopoietic system that can be directly improved by HSC transplantation, for example sickle cell anemia (Figure 1.8) [170].

Important examples of diseases that can be improved indirectly by HSC transplantation are lysosomal storage diseases (LSD). This is a group of devastating systemic diseases that are caused by gene defects in hydrolytic enzymes in lysosomes. Many of these diseases can be improved by enzyme replacement therapies, but HSC transplantation is sometimes the only effective long-term therapy. Already 1980, a child with severe Hurler syndrome was HSC transplanted, and this great success paved the way to establish HSC transplantation in several LSDs during the past 35 years [170, 178]. LSDs are improved by HSC transplantation by healthy cells that engraft to the tissues and secrete functional lysosomal enzymes that are taken up by recipient cells, leading to a metabolic correction [179].

The potential of HSC transplantation is immense, however in non-malignant conditions the risks associated with the procedure limit its application. For this reason, various reduced intensity transplantation strategies are being developed.

HSC transplantation for tolerance induction

If only the periphery is tolerized, novel alloreactive T cells will arise and eventually re-

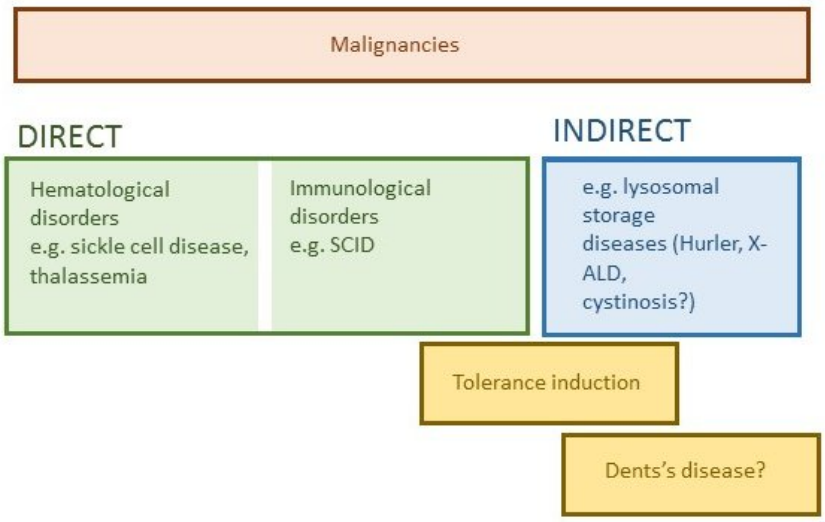

Figure 1.8: Indications for HSC transplantation: Besides malignancies, HSC transplantation is performed to directly correct diseases of the hematopoietic system and the immune system. It has also great potential in correcting diseases indirectly, e.g. it is an established procedure for treatment of several LSDs and it might potentially improve Dent's disease. HSC transplantation for tolerance induction has been successfully performed in patients, but reduced toxicity conditioning is desirable.

ject transplanted, highly immunogenic tissues like the skin. However, it is possible to re-program the immune system by transplanting HSCs derived from the organ donor. The phenomenon of central immune tolerance has been discovered 70 years ago by Owen and coworkers. They made the observation that cattle dizygotic twins display chimerism („mosaicism“) and long-term tolerance towards each other's skin grafts [180]. Experimentally, tolerance was induced by exposing mouse foetuses to homogenized allogenic tissues, an experiment that was performed by Billingham, Brent and Medawar [181].

In adults, immune tolerance can be achieved by lethal irradiation followed by reconstitution with allogeneic HSCs, leading to full chimerism. However, this treatment is very toxic due to the complete ablation of the host's immune system and the high risk for GVHD, and thus not acceptable for the purpose of tolerance induction. An elegant alternative is the induction of mixed chimerism, a state where recipient and donor HSCs co-exist (Figure 1.9) [182]. Physiological mechanisms come into play, where host- and donor-derived antigen-presenting cells (APCs) migrate to the thymus and take place in the process of central deletion

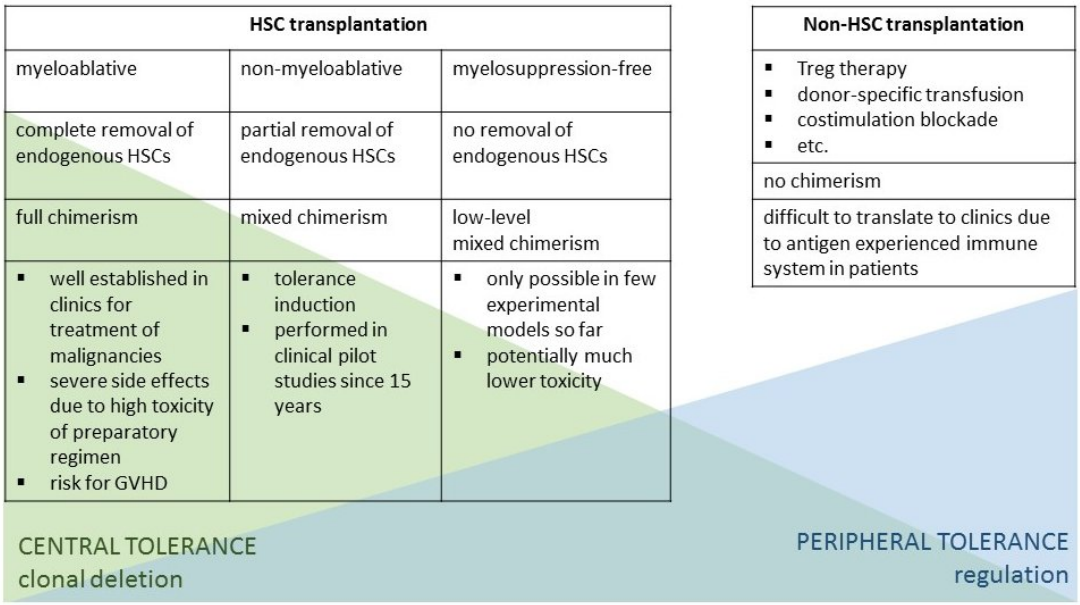

Figure 1.9: Strategies for tolerance induction: The side effects associated with myeloablative conditioning are not acceptable for the purpose of tolerance induction. Mixed chimerism induction requires less intensive conditioning of the recipient and has been successfully translated to clinics.

of self- and donor-reactive thymocytes, resulting in robust donor-specific tolerance in addition to self-tolerance [182]. However, lethal irradiation for mixed chimerism induction is unacceptable when considering clinical translation of this tolerance approach. Thus, much effort was given during the past 30 years in developing alternative protocols that allow donor BM engraftment under non-myeloablative, reduced-toxicity conditioning regimens. For successful allo-HSC transplantation, two requirements have to be met:(1) in order to prevent rejection of the transplanted BM, peripheral and thymic alloreactivity needs to blocked, (2) it is generally assumed, that there is a need to open some stem cell niche „space“ for the transplanted HSCs [183]. Allogeneic BM cells transferred into an unconditioned recipient are rapidly rejected. To block peripheral reactivity, different approaches have been proposed, e.g. T cell depleting antibodies, in some instances combined with thymic irradiation (depending on the irradiation dose of concomitant total body irradiation (TBI)), or costimulation blockade [183–186]. When using costimulation blockade, only alloreactive T cells undergo peripheral deletion, which is clearly an advantage compared to the complete deletion of the

T cell pool obtained with anti-T cell antibodies [187]. Regarding the second point, there exists however some controversy. Most conditioning protocols involve sublethal TBI (between 1.5 and 6 Gy) or cytotoxic drugs. This is thought to remove some endogenous HSCs and create space for the transplanted HSCs, while additionally having an immunosuppressive effect. However, it is possible to omit myelosuppression under certain conditions: e.g. by administrating „mega“ doses of BM cells (200x 10^6) under costimulation blockade [188], or MR1 in combination with sirolimus or CsA plus 4x 10^6 BM cells [189], or Tregs together with MR1, CTLA-4 Ig, sirolimus and 2x 10^6 BM cells [190].

We have recently developed a novel myelosuppression-free strategy to induce allogeneic chimerism and robust tolerance by pharmacological apoptosis modulation in peripheral lymphocytes [191]. By combining a two-week treatment of ABT-737 and CsA with one injection of MR1 at the time of BM infusion (25x 10^6 cells), we were able to induce stable mixed chimerism (Figure 1.10). ABT-737 tolerized the peripheral T cell pool by several mechanisms. It reduced the precursor frequency of allo-reactive T cells by unspecific deletion of peripheral T cells. Further, it boosted the function of pro-apoptotic Bim, which is required for efficient deletion of allo-reactive T cells under MR1 costimulation blockade [191]. As described later in the Results section, Tregs are also important players in ABT-737 mediated tolerance induction. The mechanisms that allow engraftment of allogenic HSCs under this myelosuppresion-free situation is under investigation.

As outlined above, clinical translation of tolerance strategies has proven difficult because of the presence of allo-reactive Tm. Furthermore, many pre-clinical protocols rely on blocking anti-CD154 signalling. However, blockade of this pathway resulted in thromboembolic events in non-human primates [192]. Thus, of the many tolerance induction strategies reported in mice, only few were successful in large animals and solely two (a third one is questioned) mixed chimerism approaches are used for renal allograft tolerance induction in patients. These protocols were developed initially in the context of hematologic malignancies and later used solely for tolerance induction.

The Stanford group around Samuel Strober has developed a successful protocol to transplant HLA-matched G-CSF mobilized HSCs. Conditioning is based on total lymphoid irradiation (TLI) and antithymocyte globulin (ATG), plus immunosuppression with CsA for 6 months, my-

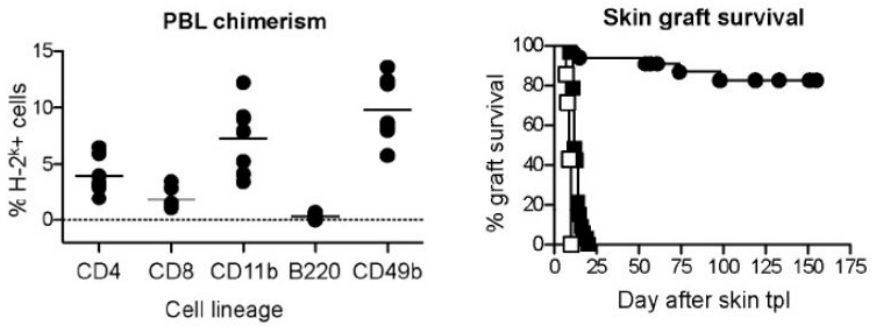

Figure 1.10: Myelosuppression-free mixed chimerism induction with a two-week ABT-737 treatment, low-dose CsA, MR1 and the transplantation of 25x 10^6 BM cells. The low chimerism levels obtained with this protocol were sufficient to promote robust skin graft tolerance.
Adapted from Cippà, Gabriel et al. [191]

cophenolate mofetil for one month and prednison [193]. In 16 out of 22 patients, persistent chimerism for more than 6 months was established and these patients could be completely weaned from immunosuppression without graft loss and signs rejection (Figure 1.11) [194].

Another group at Massachusetts General Hospital around David Sachs and Megan Sykes has also successfully developed a clinical tolerance protocol. This protocol includes induction with cyclophosphamide, ATG, thymic irradiation, and immunosuppression with CsA for 2-3 months for the transplantation of HLA-matched stem cells [195]. For HLA-mismatched stem cells, the induction protocol includes rituximab (anti-CD20 mAB) and siplizumab (anti-CD2 mAB) instead of ATG, prolonged CsA for 9 months and short-term prednisone [196]. In an initial study, patients with renal failure secondary to multiple myeloma received HLA-matched stem cells, which resulted in long-term kidney graft acceptance in most patients [195]. Following HLA-mismatched stem cell transplantation, only transient chimerism was achieved, which was however sufficient to induce long-term allograft survival and function (>11 years until now) [196].

Taken together, these clinical trials represent a proof-of-principle that tolerance induction in organ recipients is an achievable goal. However, its application is limited to living-donor organ recipients. Given the fact that nowadays rejection can be well controlled with immunosuppressive drugs, it is difficult to incorporate such a novel approach

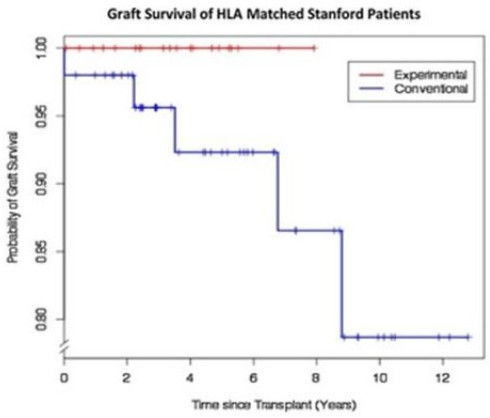

Figure 1.11: HLA-matched kidney graft survival
Patients were either enrolled in the Stanford tolerance protocol for mixed chimerism induction (20 patients, red) or they underwent standard immunosuppressive treatment in Stanford (49 patients, blue). The Kaplan-Meier curve shows death censored graft survival.
Adapted from Scandling et al. [194]

into clinical practice. And it is further complicated as there are still some safety issues to be resolved (e.g. profound leukopenia). Efforts to further reduce toxicity of the conditioning regimen are needed for a broad use of HSC transplantation in benign conditions.

HSC transplantation for the correction of kidney diseases

The use of stem cells in the regenerative and therapeutic setting has high potential, and thus receives a lot of attention from the research community. Many studies have suggested that BM-derived stem cells display plasticity and differentiate into cells of other lineages such as myocytes, endothelium, hepatocytes, neurons and glia [197–201], while other studies present evidence for fusion of these cells with other cell types [202–205]. However, transdifferentiation of both, HSCs and MSCs, is questioned by experts because such studies often cannot be reproduced by other laboratories, and results may arise from false detection methods (e.g. superposition of cells on microscopic slides).

Based on their reparative and angiogenic properties, BM-derived stem cells are considered an attractive therapy for renal repair after injury or for the therapy of renal diseases. Especially MSCs have been shown to prevent or attenuate acute kidney injury such as ischemia/reperfusion injury (I/R injury), cisplatin-induced acute kidney injury or anti-Thy1.1 glomerulonephritis [173, 206–208]. In these instances, MSCs were systemically or locally infused, which reduced acute injuries by paracrine or endocrine mechanisms rather than by engraftment. HSCs have been shown to improve recovery upon acute renal injury [209–211]. However, all the mentioned studies have used animal models with „artificial“ kidney injuries and thus might be of limited

translational importance.

In contrast, a fascinating study by Syres et al. has presented evidence that stem cells can correct a tubulopathy in a clinically relevant cystinosis model [212]. Cystinosis is a hereditary LSD that is caused by defects in the *CTNS* gene that encodes a cystine transporter (cystinosin) located in lysosomes [213, 214]. Upon defects in cystinosin, cysteine accumulates in lysosmes, which leads to the formation of cysteine crystals that cause organ-specific damage. The kidneys are severely affected by the mutation, and patients show generalized proximal tubular dysfunction (Fanconi syndrome) and progress to end-stage renal disease in childhood [215]. In the above mentioned study, Ctns KO mice, which recapitulate well the disease phenotype of cystinosis, were transplanted with syngeneic WT BM cells, and cystine content in organs and renal function were measured twice in four months. The authors show engraftment of transplanted BM-derived cells in different organs along with a marked reduction of cysteine crystals. In the kidneys of Ctns KO mice, transplanted cells were located in the interstitial space, but only a few of the cells were of lymphoid or macrophage lineage. Moreover, cells were found in close proximity and in co-localisation with proximal and distal tubular cells. Regarding kidney function, treated mice displayed reduced creatinine and urea levels in their plasma, while creatinine clearance, serum phosphate and alkaline phosphatase did not significantly improve. Furthermore, proteinuria and urinary phosphate remained unchanged [212].

Improvements of chronic kidney injuries in LSDs upon HSC transplantation have been shown before [216, 217]. However, this study is important by the fact that cystinosis is caused by a defect in a membrane-bound transporter in lysosomes of epithelial cell. Thus, in contrast to other LSDs where probably functional enzymes are secreted by donor-derived cells and then taken up by recipient cells, the mechanism of action in this case has to be different. It is important to test whether the findings of Syres et al. can be expanded to other kidney diseases that are caused by defects of membrane-bound transporters.

1.4 Dent's disease

The renal Fanconi syndrome is characterized by the loss of substances that pass the glomerular filtration barrier, such as low molecular weight (LMW) proteins, including albumin and transferrin, enzymes, hormones, and substances such as glucose and electrolytes [219, 220]. The massive loss of substances in the urine is caused by a generalized dysfunction of renal proximal tubuli (PT) cells. The reabsorption of filtered substances requires an extensive endocytic apparatus in PT cells. Many proteins (e.g. albumin, hemoglobin, apolipoproteins) are reabsorbed by clathrin-mediated endocytosis, a process that involves binding to the megalin and cubilin scavenger receptors present at the apical brush border membrane, followed by internalization of the receptor/protein complex and dissociation in acidified endosomes/lysosomes [221]. The scavenger receptors are recycled back to the membrane, while the proteins are subjected to lysosomal degradation. Defects in the endosomal and/or lysosomal pathway prevent this recycling of megalin and cubilin, and thus often result in the clinical manifestations of Fanconi syndrome.

ClC-5 is considered to play a central role in orchestrating receptor-mediated uptake of filtered substances in the kidneys. It is an electrogenic 2Cl-/H+ exchanger that is mainly localized in intracellular vesicles of PT epithelial cells [222] and exploits the H+ gradient generated by V-ATPase (a proton pump) to move Cl- ions into endosomes (Figure 1.12) [223]. Mice with inactivating mutations of ClC-5 show severe defects in endocytosis due to loss of the multiligand receptors megalin and cubilin at the brush border membrane, and impaired lysosomal processing of internalized ligands [224]. The exact mechanism leading to this defective PT endocytosis in ClC-5 KO mice is not completely understood to date [219], but it has been shown that impaired Cl- flux and a lack of endosomal acidification both cause Fanconi syndrome [225, 226].

Dent's disease is rare X-linked congenital disorder, which is caused in approximately 60% of affected males by inactivating mutations in the *CLCN5* gene [227]. Patients show a similar disease phenotype as ClC-5 KO mice, including renal Fanconi syndrome with loss of LMW proteins, calcium, amino acids, phosphate, potassium and glucose in the urine. LMW proteinuria involves the loss of clara cell protein (CC16), α1 and β2 microglobulins, retinol-binding protein and vitamin D binding protein, and it is the most consistent manifestation of Dent's disease [228]. Secondary to loss of all these sub-

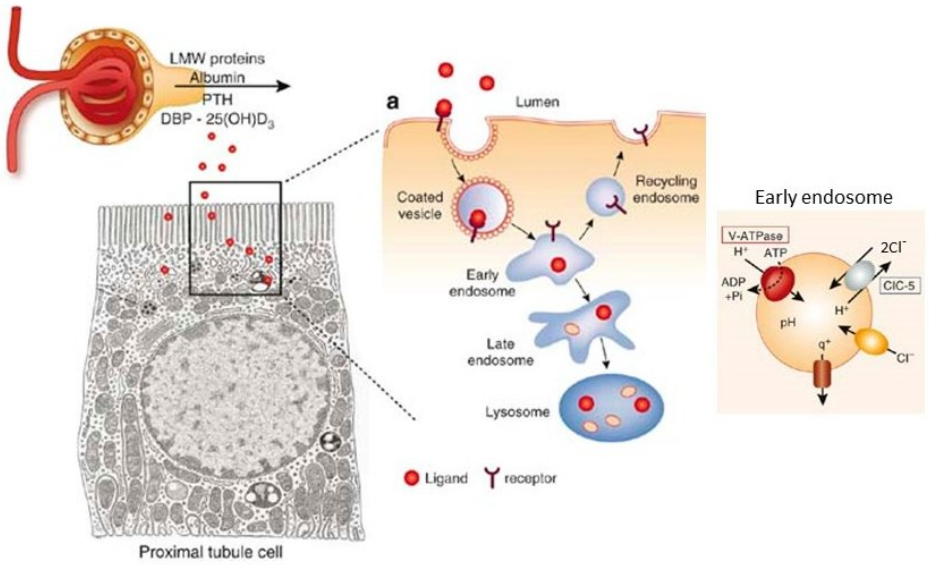

Figure 1.12: Dent's diseases is caused by defective receptor-mediated endocytosis in PT cells due to mutations in the ClC-5 exchanger in early endosomes.
Adapted from Devuyst and Pirson [218].

stances, patients may suffer from rickets, osteomalacia, kidney stones or calcifications, or vitamin A deficiency due to loss of retinol-binding protein in the urine [228–230]. Between the age of 30 to 50 years, patients may progress to end-stage renal disease, requiring dialysis or renal transplantation [229]. There is no curative treatment for Dent's disease, thus care of patients is solely supportive with focus on the prevention of nephrolithiasis [228].

The encouraging results obtained with BM transplantation in the pre-clinical mouse model of cystinosis prompted us to question whether this might also be a treatment option for Dent's disease.

2 Experimental Studies

2.1 Bcl-2 inhibition to overcome memory cell barriers in transplantation

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Abstract

Memory T cells (Tm) represent a major barrier for immunosuppression and tolerance induction after solid organ transplantation. Taking into consideration the critical role of the intrinsic apoptosis pathway in the generation and maintenance of Tm, we developed a new concept to deplete alloreactive Tm by targeting Bcl-2 proteins. The small-molecule Bcl-2/Bcl-xL inhibitor ABT-737 efficiently induced apoptosis in alloreactive Tm in vitro and in vivo and prolonged skin graft survival in sensitized recipients. A short course of ABT-737 induction therapy prevented Tm-mediated resistance in a donor-specific transfusion model and allowed mixed chimerism induction across Tm barriers. Since Bcl-2 inhibitors yielded encouraging safety results in cancer trials, this novel approach might represent a substantial advance to prevent allograft rejection and induce tolerance in sensitized recipients.

Introduction

The translation of experimental therapies into the clinic inevitably depends on the ability to understand and overcome biological differences between laboratory animals and humans. The memory T cell (Tm) compartment has been recognized as a major hurdle for the translation of immunomodulatory therapies [231]: the adaptive immune system of laboratory mice - young animals that have barely been exposed to pathogens - mostly consists of naïve lymphocytes, whereas in large animals and humans, a progressive shift from naïve to memory lymphocytes occurs during life, after exposure to pathogens and as a result of a progressive thymic function decline [232, 233]. Tm are less dependent on costimulation [234] and are less efficiently inhibited by regulatory T cells [235]. This has broad clinical implications for solid organ transplantation, since Tm generated in response to pathogens can react against alloantigens in a process called heterologous immunity [150]. Standard immunosuppressive agents and lymphocyte depleting antibodies have a limited impact on memory cells [236], and the efficacy and safety of new strategies to inhibit Tm by targeting adhesion molecules such as CD2 [237] and LFA-1 [238] remain to be proven. Therefore, innovative and effective strategies to inhibit or deplete Tm are required.

Apoptosis, and particularly the regulation of the intrinsic apoptosis pathway by pro- and anti-apoptotic factors of the Bcl-2 family, is critically involved in the physiological T cell contraction at the end of the immune response, in the selection of T cell clones for memory generation and in Tm maintenance. More precisely, the balance between the pro-apoptotic Bim and the anti-apoptotic Bcl-2 and Bcl-xL controls Tm survival [60, 66, 239, 240], and a direct inhibition of Bcl-2 and Bcl-xL reduced the number of T cells with a memory phenotype in mice [241]. Therefore, we hypothesized that a pharmacological modulation of the intrinsic apoptosis pathway using recently developed pro-apoptotic small molecule Bcl-2 inhibitors, such as ABT-737 and ABT-263 (navitoclax), might represent a promising opportunity to control memory T cell responses [97, 98, 242, 243]. The application of this approach in transplantation medicine seems particularly promising, since Bcl-2 inhibitors suppressed allogeneic immune responses and promoted the induction of donor-specific tolerance in combination with costimulation blockade [191, 244].

Materials and Methods

Mice

C57BL/6 (B6, H-2^b), CBA (H-2^k), BALB/c (H-2^d) and BM3.3 (CBA, H-2^k) mice were housed in specific pathogen-free conditions at the University of Zürich. The BM3.3 mouse, which expresses on all CD8 T cells a transgenic T cell receptor (TCR) selective for a naturally processed octapeptide bound to the allogeneic MHC class I molecule H-2^b, was kindly provided by A.-M. Schmitt-Verhulst [245, 246]. All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office, Canton of Zürich, Switzerland).

Fluorescence activated cell sorting (FACS)

FACS analyses were performed with a BD-FACSCanto II (Becton Dickinson, Basel, CH). Anti-mouse CD3-FITC, CD3-PE-Cy7, CD4-PE, CD4-FITC, CD8-APC, CD8-APC-Cy7, CD25-FITC, CD25-PE, CD25-APC, CD44-FITC, CD44-PE-Cy7, CD62L-PE, CD62L-APC, CD69-FITC, IFN-γ-FITC and propidium iodide (PI) were purchased from eBioscience (Frankfurt, Germany). Intracellular IFN-γ staining was performed according to manufacturer's instructions (fixation and permeabilization wash buffers from BioLegend, Lucerne, Switzerland). BM3.3 CD8 T cells were detected using the clonotypic antibody Ti98, which selectively binds to the BM3.3 TCR and was kindly provided by A.-M. Schmitt-Verhulst [247]. A secondary rat anti-mouse IgG-PE antibody was purchased from Becton Dickinson (Basel, Switzerland). Chimerism was analyzed in white blood cells at different time points after bone marrow transplantation. Background signal measured in a naïve CBA mouse was subtracted to determine the % of donor-derived cells. Donor-derived cells were identified by anti-H-$2K^b$-FITC (Becton Dickinson, Basel, Switzerland cells.

Mixed lymphocyte reaction (MLR)

MLRs were performed in 96-well plates with responder splenocytes stimulated by CD3- or CD8-depleted splenocytes from donor, 3rd party or syngeneic mice at a final concentration of 4 x10^6 cells/ml in culture medium containing 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 µg/ml, 2-mercaptoethanol 50 µM. For IFN-γ detection, responder cells from memory mice were stimulated with CD3-depleted splenocytes and 10 ng/ml rIL-2 (RnD systems Inc., Minneapolis, USA) for 15h. Brefeldin A (10 µg/ml) was added to cultures for the last 3h of stimulation. T cell proliferation was measured by incorporation of ^{3}H-thymidine (Perkin Elmer, Waltham, USA) added to the culture on day 4 after stimulation. All cell separations were performed by

automatic magnetic cell sorting using an autoMACS pro separator according to the protocols of MiltenyiBiotec (BergischGladbach, Germany).

Generation of alloreactive memory cells
In vitro, BM3.3 splenocytes were stimulated with CD8-depleted B6 splenocytes in tissue-culture flasks with culture medium. Two days later, CD8 T cells were positively selected by magnetic cell sorting, washed and cultured in fresh tissue-culture flasks in the same medium supplemented with 20 ng/ml rIL-15 or rIL-2 (RnD systems Inc., Minneapolis, USA) for additional 5 days [248]. In vivo, Tm were generated either by skin transplantation or by i.v. injection of 10^7 allogeneic splenocytes as indicated.

ABT-737 sensitivity in vitro
Splenocytes or in vitro generated memory cells were cultured in 96-wells plates at a final concentration of 4 x 10^6 cells/ml in the presence of different concentrations of ABT-737 or vehicle. ABT-737 was dissolved in DMSO at a concentration of 5 mM and then diluted in culture medium. After 12h incubation, cell viability was assessed by PI exclusion in FACS, and % of values measured in corresponding vehicle-exposed cultures was calculated. ABT-737 was provided by Abbvie Bioresearch (Worcester, USA).

Quantitative RT-PCR
Reverse transcription and qPCR were performed as reported earlier [249]. Predeveloped TaqMan reagents were used for mouse Bcl-2 (Mm00477631 m1), Bcl-XL (Mm00437783 m1), Mcl-1 (Mm01257352 g1) and for the housekeeper gene 18S rRNA (Applied Biosystems Europe, Rotkreuz, Switzerland). For mouse A1, the following oligonucleotide primers and probe were designed to simultaneously detect Bcl-2A1a, Bcl-2A1b and Bcl-2A1d: sense primer 5'-ATG GAG GTT GGG AAG ATG G-3', anti-sense primer 5'-GAG CCA AGG TTC TCT CTG GTC-3', fluorescence-labelled probe (FAM) 5'-GGC TGG CTG ACT TTT CTG CAG ATG A-3'. The expression of candidate genes in alloantigen-stimulated cells of culture was normalized by 18S rRNA.

Skin grafting, donor specific transfusion (DST), bone marrow transplantation and in vivo treatment
For skin transplantation, mice were shaved and anesthetized with ketamine/xylazine. Full thickness tail skin (about 1 cm^2) was grafted and considered rejected when $<10\%$ of the graft remained viable. DST was performed by i.v. injection of 10^7 splenocytes. Bone marrow was transplanted by tail vein injection of 50 x10^6 fully MHC-mismatched B6 bone marrow cells into CBA recipients. Hamster anti-mouse CD154 (MR1, Bio-X-

cell, West Lebanon, NH, USA) was administered i.p. as indicated. For in vivo application, ABT-737 was dissolved in polyethylene glycol, Tween 80, dextrose solution and DMSO and injected i.p. at 50 mg/kg.

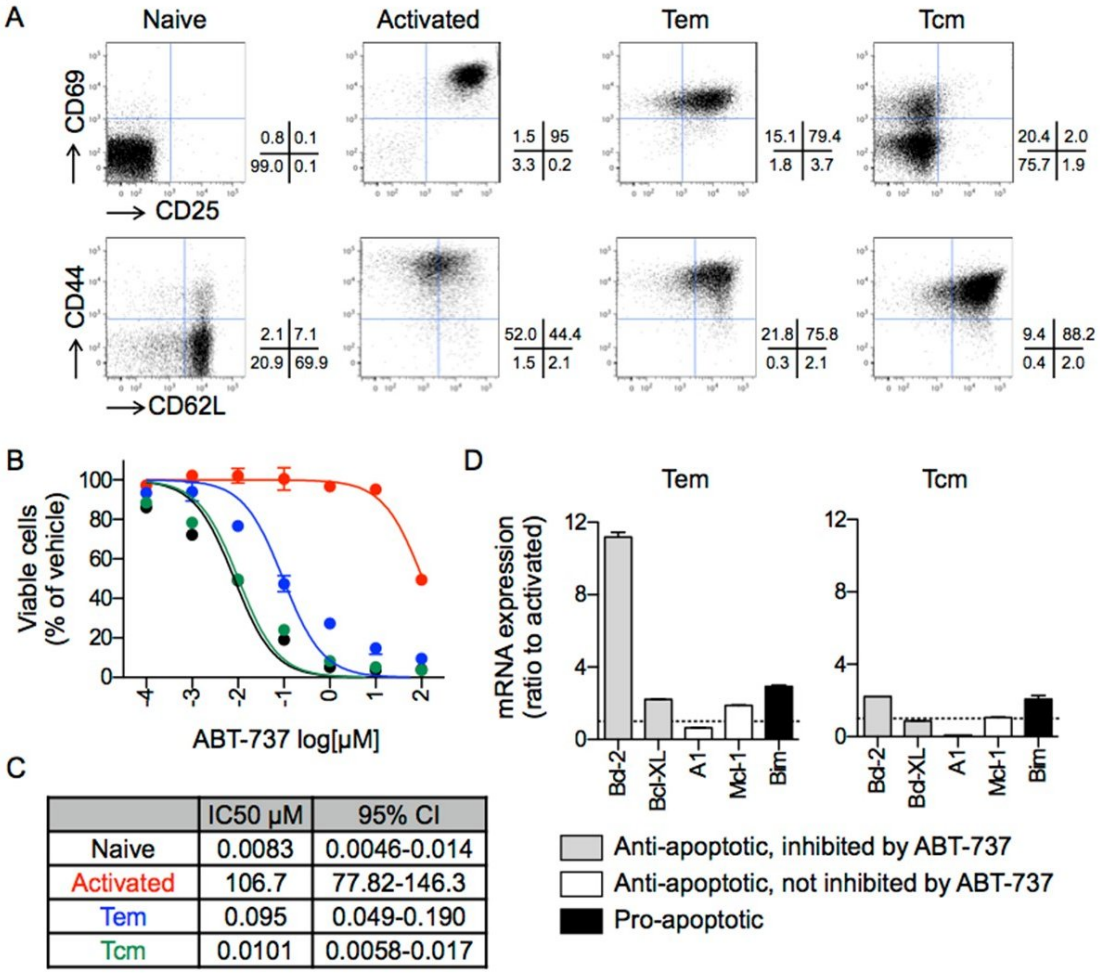
Statistics

Student t-test or Mann-Whitney test were used to compare values between groups. Half maximal inhibitory concentration (IC50) was calculated using a log(inhibitor) vs. response model. Skin graft survival was compared using log-rank test. $P<0.05$ was considered significant. Graph Pad Prism Software Version 5.0 was used for calculations.

Results

Bcl-2/Bcl-xL inhibition induces apoptosis in alloreactive memory T cells

To evaluate the sensitivity of alloreactive Tm to Bcl-2/Bcl-xL inhibition we first established an in vitro model to generate alloreactive CD8 Tm by modifying a previously reported protocol [248]. BM3.3 CD8 T cells, which express a transgenic TCR specific for the MHC class I molecule H-2K^b and can be detected with the monoclonal antibody Ti98 [245, 246] were stimulated with CD8-depleted B6 splenocytes in a classical MLR. After 2 days of culture, BM3.3 CD8 T cells were positively selected by magnetic cell sorting and further cultured in the presence of IL-15 to generate central memory T cells (Tcm) or IL-2 to generate effector memory T cells (Tem). After 5 days, we obtained a homogeneous population of CD8+Ti98+ cells with the phenotypic characteristics of Tcm (CD25-CD69-CD44+CD62L+) or Tem (CD25+CD69+CD44+) (Figure 2.1 A). We compared the sensitivity to ABT-737 in Tm with activated T cells (2 days of MLR without additional cytokine incubation) and naïve T cells, by measuring cell viability by propidium iodide (PI) exclusion in FACS after 12h of incubation with different concentrations of ABT-737 (Figure 2.1 B-C). According to our previous reports [61], CD8 T cells were resistant to ABT-737 induced apoptosis in the first days after allogeneic stimulation. This correlated with the increased expression of Bcl-2A1, an anti-apoptotic factor not inhibited by ABT-737. In contrast, in vitro generated central memory CD8 T cells were as sensitive to ABT-737 as naïve CD8 T cells, with apoptosis induction in nanomolar ABT-737 concentrations. Tem were slightly but significantly more resistant to ABT-737 than naïve cells or Tcm. This correlated with the expression of anti-apoptotic Bcl-2 factors. In both, Tcm and Tem, we observed a progressive down-regulation of the resistance factor Bcl-2A1 as compared to early-activated cells. In con-



	IC50 µM	95% CI
Naive	0.0083	0.0046-0.014
Activated	106.7	77.82-146.3
Tem	0.095	0.049-0.190
Tcm	0.0101	0.0058-0.017

Figure 2.1: In vitro generated alloreactive Tm are sensitive to ABT-737
BM3.3 splenocytes were stimulated with CD8-depleted B6 splenocytes. Two days later, activated CD8 T cells („activated") were positively selected by MACS and further cultured in the presence of IL-15 to generate Tcm or IL-2 to generate Tem. **(A)** Phenotype characterization of naïve T cells, activated T cells, Tem and Tcm. **(B-C)** After incubation with different concentrations of ABT-737 or vehicle for 12h, cell viability was assessed by PI exclusion in FACS (duplicates, one representative experiment of two is shown, IC50: half maximal inhibitory concentration, CI: confidence interval). **(D)** After 5 days of IL-2 or IL-15 incubation, cells were lyzed for mRNA isolation. The expression profile of Bcl-2 family genes was assessed by RT-PCR. Relative gene expression compared to early activated cells is shown (one representative experiment of two is shown).

trast, expression of Bcl-2, which is inhibited by ABT-737, was increased after 5 days of incubation with IL-2. The expression of the anti-apoptotic factors Bcl-xL and Mcl-1 and of the pro-apoptotic factor Bim was similar in early-activated T cells, Tem and Tcm (Figure 2.1 D). These results were validated ex vivo. CBA mice were primed with an injection of B6 splenocytes. Eight weeks after priming ABT-737 sensitivity in different T cell populations was assessed in vitro by measuring viability in FACS after 12h of incubation with ABT-737. Naïve (CD44-) and memory (CD44+) CD4 or CD8 T cells displayed a similar sensitivity to ABT-737 (suppl. Figure 2.6). Consistently, in primed mice treated with ABT-737 we observed a parallel depletion of naïve, Tcm and Tem with a statistically non-significant increase of Tem in mice exposed to ABT-737 compared to vehicle (data not shown). Thus, Bcl-2 and Bcl-xL, which are selectively inhibited by ABT-737, represent a potential molecular target to deplete Tm. The relevance of these findings was further investigated at the functional level.

Bcl-2/Bcl-xL inhibition suppresses allogeneic memory T cell responses

Allogeneic polyclonal memory recall responses were investigated ex vivo and in vivo. In a first experiment, CBA mice were primed with B6 skin grafts. Four months later recipient mice were treated with ABT-737 or vehicle for 5 days and then euthanized. Their splenocytes were stimulated in vitro overnight with T cell depleted syngeneic (CBA), donor-derived (B6) or 3rd party (BALB/c) splenocytes. Donor-reactive CD8 T cells were detected by intracellular IFN-γ staining in FACS (Figure 2.2 A). For quantitative analysis, the percentage of CD8+ IFN-γ+ cells among T cells after allogeneic stimulation was divided by the value measured after syngeneic stimulation. Compared to vehicle treated memory mice, the 5-day ABT-737 treatment inhibited donor-specific memory recall response, as shown in a 40% lower ratio of IFN-γ positive cells. In contrast, ABT-737 treatment did not influence the response against 3rd party stimulators (Figure 2.2 B). Thus, the depleting effect of ABT-737 on Tm in vivo was sufficient for a significant inhibition of memory recall responses. This finding was confirmed in a skin graft model. B6 mice were primed with BALB/c skin and 7 weeks later re-transplanted with BALB/c skin under treatment with ABT-737 or vehicle, starting 5 days before transplantation. According to our prior observations [61], ABT-737 did not significantly inhibit rejection in naïve recipients in a fully MHC-mismatched combina-

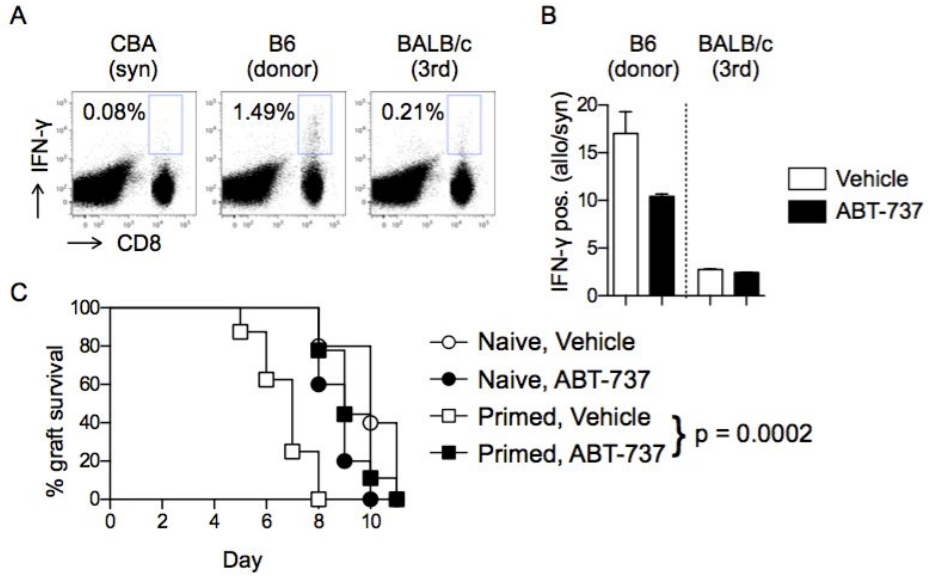


Figure 2.2: Inhibition of allogeneic memory recall responses by ABT-737
(A) CBA mice were primed with B6 skin. Four months later, splenocytes were stimulated in vitro with T cell depleted CBA (syn), B6 (donor) or BALB/c (3rd party) cells. FACS plots of 3 representative examples are shown, indicating the percentages of CD8+ IFN-γ+ cells among T cells after overnight stimulation. **(B)** For a quantitative analysis, the % of CD8+ IFN-γ+ cells among T cells after allogeneic stimulation was divided by the value measured after syngeneic stimulation (allo/syn). Values obtained using cells isolated from mice treated for 5 days with ABT-737 or vehicle before splenocyte isolation are shown (n=2). **(C)** Seven weeks after priming with BALB/c skin grafts, B6 recipients were re-transplanted with BALB/c skin on the contra-lateral site. ABT-737 or vehicle were administered starting on day 5 before transplantation with daily i.p. injections. Skin graft survival in primed mice treated with ABT-737 or vehicle was compared with the results obtained in naïve mice (n=5-9 /group, 2 independent experiments).

tion. However, ABT-737 significantly prolonged skin graft survival in primed mice, resulting in a similar skin graft survival in primed mice treated with ABT-737 and in naïve mice (Figure 2.2 C).

Since the regulation of the intrinsic apoptosis pathway is not only critically involved in Tm maintenance, but also in Tm generation during the contraction phase at the end of the immune response [66, 239], we hypothesized that exposure to ABT-737 during priming might influence later memory recall responses in primed recipients. Naïve B6 mice were transplanted with BALB/c skin grafts and subsequently treated with ABT-737 or vehicle for 14 days. Four months later, recipients were euthanized and splenocytes were isolated for further in vitro experiments. Re-stimulation of splenocytes isolated from recipient mice treated with ABT-

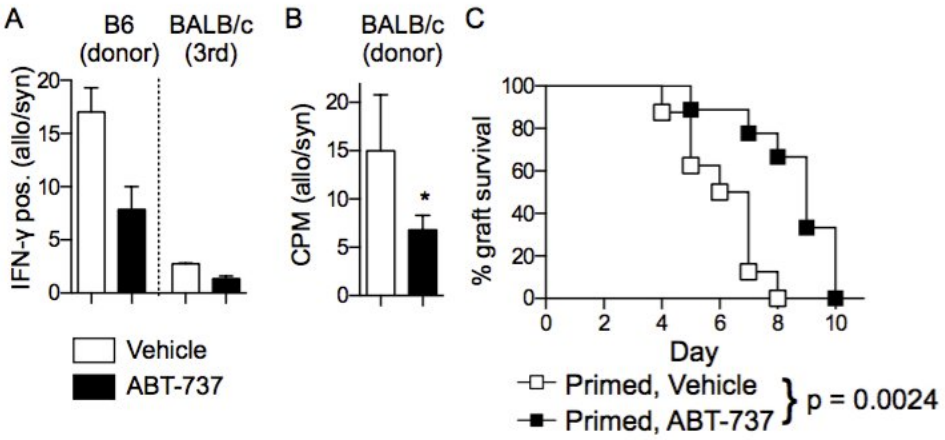
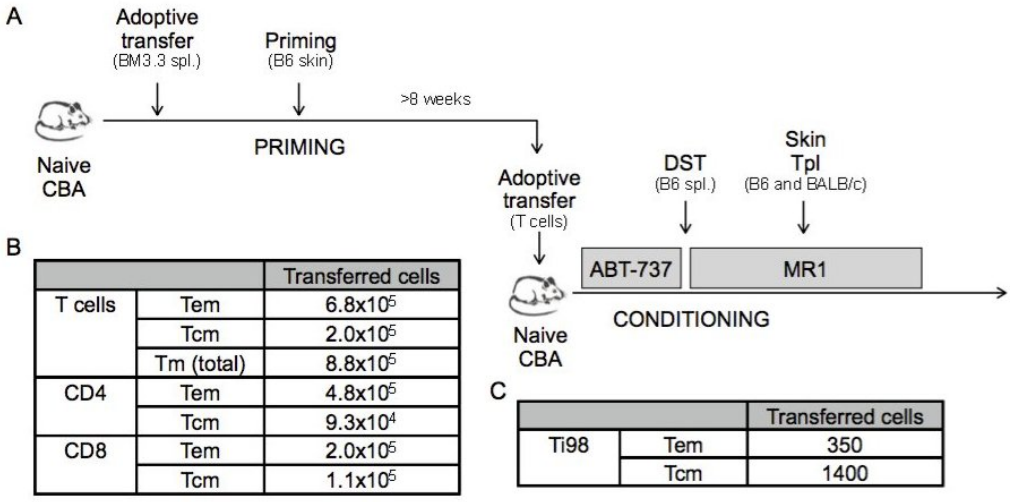


Figure 2.3: ABT-737 during priming inhibits memory recall responses
(A) CBA mice were transplanted with B6 skin grafts and immediately treated with ABT-737 or vehicle for 14 days. Four months after priming, recipients were euthanized and their splenocytes re-stimulated in vitro. For a quantitative analysis the % of CD8+ IFN-γ+ cells among T cells after allogeneic stimulation was divided by the value measured after syngeneic stimulation (allo/syn). Values obtained using cells isolated from mice treated with ABT-737 or vehicle during priming are shown (n=2). **(B)** Cell proliferation was assessed by incorporation of ^{3}H-Thymidine in the same experimental setting, but using cells isolated from B6 mice previously primed with BALB/c skin grafts under the effect of ABT-737 or vehicle. Counts per minute measured using cells isolated from mice treated with ABT-737 or vehicle during priming are shown (allo/syn, *$p<0.05$, n=5). **(C)** B6 mice were transplanted with BALB/c skin grafts and immediately treated with ABT-737 or vehicle for 14 days. After more than 6 months, the same recipients were transplanted with a second BALB/c skin graft without additional treatment. Mean skin graft survival was 6 days in mice treated with vehicle and 9 days in mice treated with ABT-737 during priming (pooled data from two independent experiments, n=8-9 /group).

737 during priming, showed a 52% reduction in the number of IFN-γ positive cells as compared to vehicle treated mice (Figure 2.3 A). Similarly, exposure to ABT-737 significantly reduced proliferation after re-stimulation in vitro in the same experimental setting, but in a different mouse strain combination (Figure 2.3 B, B6 recipients, BALB/c donors). Moreover, secondary skin transplantation performed several months after priming without any additional treatment resulted in a modest but statistically significant prolonged skin graft survival in mice treated with ABT-737 during priming (Figure 2.3 C). Thus, ABT-737 treatment during or late after priming significantly inhibited allogeneic memory immune responses in a stringent, fully MHC-mismatched skin graft model.

Bcl-2/Bcl-xL inhibition restores sensitivity to costimulation blockade

Tm are notoriously less dependent on costim-



		Transferred cells
T cells	Tem	$6.8x10^5$
	Tcm	$2.0x10^5$
	Tm (total)	$8.8x10^5$
CD4	Tem	$4.8x10^5$
	Tcm	$9.3x10^4$
CD8	Tem	$2.0x10^5$
	Tcm	$1.1x10^5$

		Transferred cells
Ti98	Tem	350
	Tcm	1400

Figure 2.4: continued on next page

ulation than naïve T cells, and therefore represent an important barrier for immunomodulatory strategies based on the inhibition of CD28/CD80/CD86 or CD154/CD40 signalling [234]. We tested the possibility to overcome this obstacle by Bcl-2/Bcl-xL inhibition in a model including DST, costimulation blockade with anti-CD154 (MR1) and skin transplantation [132]. To selectively investigate the effect on Tm while excluding the effect of pre-existing donor-reactive antibodies, an adoptive transfer approach was chosen (Figure 2.4 A). In a first step, CBA mice were adoptively transferred with 10^7 BM3.3 splenocytes and primed with B6 skin grafts. BM3.3 derived CD8 T cells, which express a transgenic TCR specific for the MHC class I molecule H-$2K^b$ in a CBA background and can be detected with the monoclonal antibody Ti98, were used to track a defined population of donor-reactive T cells along the experiment. Eight weeks after priming, recipient mice were euthanized and T cells were isolated by non-touch magnetic cell sorting and transferred into naïve CBA recipients. A total of 5x 10^6 cells/mouse were transferred, including about 800'000 T cells with a memory phenotype (Figure 2.4 B). About 2'000 of the adoptively transferred cells were Ti98+ and >85% of these transgenic cells had a memory phenotype (Figure 2.4 C). After adoptive transfer, recipient mice received a 2-day induction therapy with ABT-737. The following day (day 0), mice were injected with MR1 and donor-type (B6) splenocytes (DST). MR1 (0.25

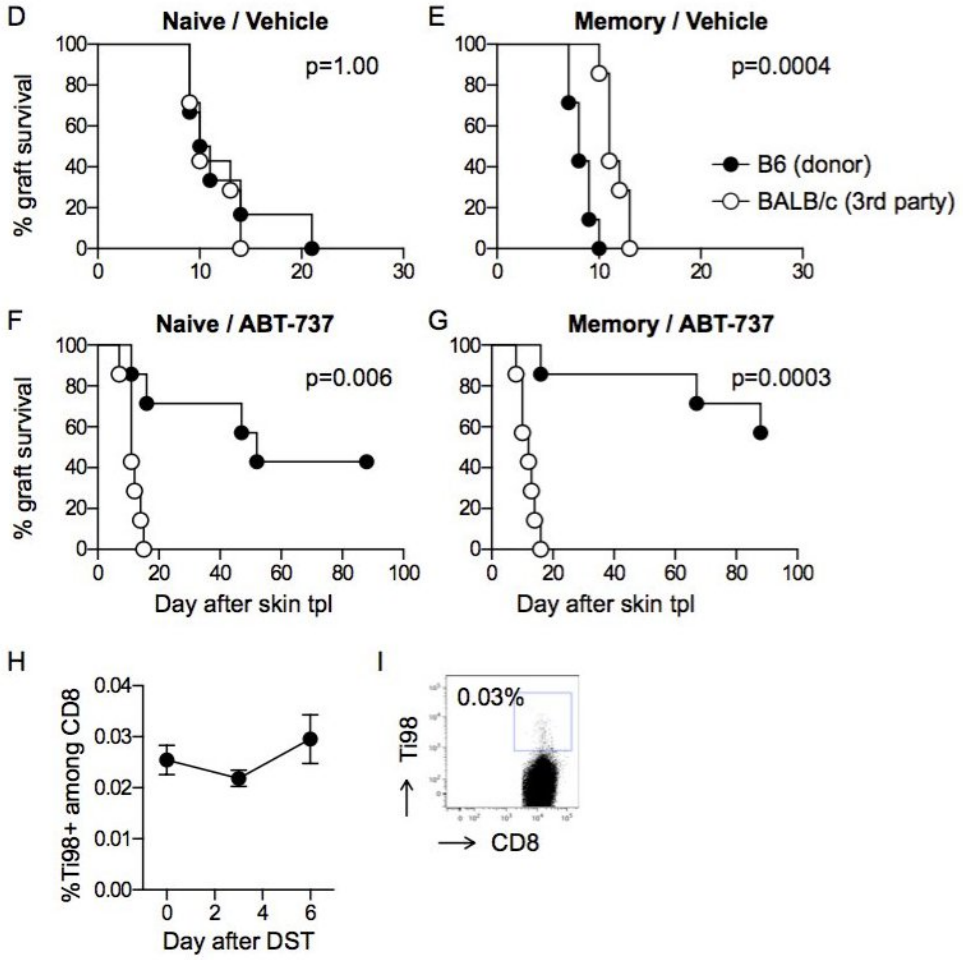


Figure 2.4: ABT-737 restores sensitivity to costimulation blockade after adoptive transfer of Tm

(A) Experimental setup: naïve CBA mice were transferred with syngeneic BM3.3 splenocytes and primed with B6 skin grafts. After 14 weeks, the primed mice were euthanized, T cells isolated from the spleen by non-touch T cell MACS sorting and transferred into naïve CBA mice. Control mice did not receive additional cells. Induction therapy with ABT-737 (5 injections of 50 mg/kg ABT-737 over 2 days) was started on the same day. The recipients received DST (10^7 B6 splenocytes), and 7 days later skin transplantation from B6 and BALB/c donors was performed. Anti-CD154 (MR1, 0.25 mg/injection, i.p.) was administered at day 0, 3, 6, 11 and 14 after DST. Quantitative characterization of the transferred lymphocytes is reported in **(B)** for polyclonal T cells and in **(C)** for transgenic Ti98+ cells (one representative experiment of two). Survival of donor-type (B6, filled circles) and third party (BALB/c, empty circles) skin graft is shown in D–G (n=7/group): **(D)** vehicle, DST, MR1, **(E)** Tm adoptive transfer, vehicle, DST, MR1, **(F)** ABT-737, DST, MR1, **(F)** Tm adoptive transfer, ABT-737, DST, MR1. To monitor the fate of donor-specific Tm during the conditioning phase, recipient mice were euthanized on day 0, 3 and 6 after DST and the splenocytes analyzed by fluorescence activated cell sorting. The percentage of Ti98+ cells among CD8 in the spleen at different time points is reported in **(H)** (n=3). One representative example is shown in **(I)**. DST, donor-specific transfusion; Tm, memory T cells.

mg) was injected on day 0, 3, 6, 11 and 14 after DST. On day 7 after DST, donor- (B6) and third party-derived (BALB/c) skin grafts were transplanted. Naïve mice without T cell transfer rejected both skin grafts within 20 days (Figure 2.4 D). Tm transfer resulted in an accelerated donor-type skin graft rejection, indicating that transferred Tm were functional (Figure 2.4 E). Mice additionally treated with ABT-737 displayed a marked donor-specific hyporesponsiveness (Figure 2.4 F). Notably, ABT-737 completely abrogated the deleterious effect of Tm in this model (Figure 2.4 G).

To follow the fate of adoptively transferred Ti98+ cells in the same experimental setting, recipient mice were euthanized at different time points during the conditioning phase for FACS analysis. After ABT-737 conditioning and before DST (day 0) we found about 500 CD8+Ti98+ cells/spleen. ABT-737 induced lymphopenia progressively resolved in the following days, resulting in an absolute increase in the number of Ti98+ cells, but the percentage of Ti98+ cells among CD8 cells did not significantly change on day 3 and 6 after DST (Figure 2.4 H-I). Thus, ABT-737 induced a substantial, but incomplete deletion of donor-reactive memory T cells. This was sufficient to overcome Tm-mediated resistance to costimulation blockade in a DST model. A similar approach was tested in a mixed chimerism protocol.

Bcl-2/Bcl-XL inhibition allows mixed chimerism induction across memory barriers
A combined transplantation of a solid organ and hematopoietic stem cells to induce mixed chimerism is the only strategy to induce immunological tolerance that has found a clinical application so far [250, 251]. Costimulation blockade is a promising option to induce mixed chimerism with a reduced toxicity [188], but the translation of this approach to non-human primates and humans was particularly challenging, at least in part because of the presence of Tm [231]. We used a similar conditioning protocol to induce mixed chimerism in mice after Tm adoptive transfer as described in the previous section (Figure 2.5 A). First, CBA mice were adoptively transferred with 10^7 BM3.3 splenocytes and 2 days later primed with an i.v. injection of 10^7 B6 splenocytes. After 3 months T cells were isolated and 5x 10^6 cells were transferred into naïve CBA mice. After a 2-day ABT-737 or vehicle conditioning, recipient mice were injected with 10^7 B6 splenocytes (DST) under costimulation blockade. MR1 (0.25mg/injection, i.p.) was injected on day 0, 3, 6, 11 and 14 after DST. Seven days after DST, recipients received 50x 10^6 B6 bone marrow cells. Four

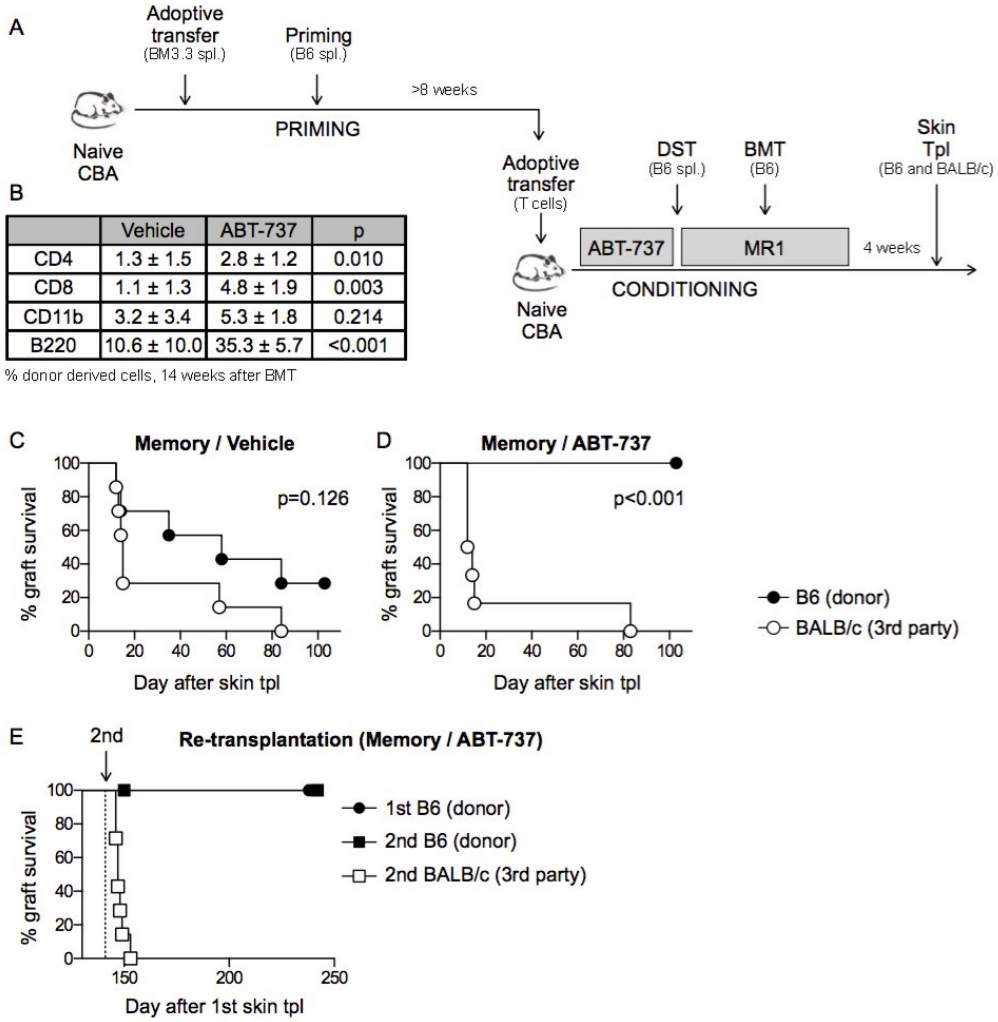
weeks later, skin transplantation from B6 and BALB/c donors was performed. Adoptive transfer of Tm impaired the efficacy of this tolerance induction protocol: the level of chimerism was very low in most recipients treated with vehicle, and both donor-type and third party–derived skin grafts were rejected (Figure 2.5 B and C). In contrast, mice additionally treated with ABT-737 after Tm transfer displayed a significantly higher level of chimerism in peripheral blood (Figure 2.5 B), and all recipients developed a donor-specific tolerance, as shown by the long-term survival of donor-derived skin grafts (Figure 2.5 D). The maintenance of a robust, systemic form of tolerance, as typically observed in mixed chimeras, was confirmed by transplantation of secondary skin grafts: all B6 grafts were accepted long term, whereas the third party controls were promptly rejected after re-transplantation (Figure 2.5 E).

Discussion

Sensitization toward HLA molecules represents an important limitation to the access to a potential organ. Several options are available to inhibit antibody-mediated rejection in sensitized recipients [252], but the therapeutic options to control Tm responses are limited. In this study, we developed a novel approach to suppress alloreactive Tm.

Fundamental studies about the role of apoptosis in Tm generation and maintenance prompted us to target the intrinsic apoptosis pathway to achieve a functionally relevant Tm depletion. Bcl-2/Bcl-xL inhibitors such as ABT-737 represent a new class of immunomodulatory drugs with a particular relevance in the field of transplantation [241]. ABT-737 inhibited allogeneic immune responses in naïve mice [244] and promoted the induction of mixed chimerism and tolerance in combination with costimulation blockade [191]. The present study demonstrates that the same approach is effective to control memory recall responses in virtue of the pro-apoptotic effect of ABT-737 on Tm.

One of the most important applications of this approach is related to the possible combination with costimulation blockade. Several studies demonstrated that the need of costimulation for T cell activation depends on intrinsic characteristics of different cell types (i.e. Tm are less costimulation-dependent than naïve T cells [234]), but also on the antigen-specific precursor frequency [253]. The marked skin graft survival prolongation observed in mice treated with ABT-737, MR1 and DST might be related to a reduced precursor frequency due to ABT-737-induced lymphopenia [243] or to the activation of regulatory mechanisms. Importantly,



	Vehicle	ABT-737	p
CD4	1.3 ± 1.5	2.8 ± 1.2	0.010
CD8	1.1 ± 1.3	4.8 ± 1.9	0.003
CD11b	3.2 ± 3.4	5.3 ± 1.8	0.214
B220	10.6 ± 10.0	35.3 ± 5.7	<0.001

Figure 2.5: Mixed chimerism and tolerance induction after Tm transfer
(A) Experimental setup: naïve CBA mice were transferred with syngeneic BM3.3 splenocytes and primed with B6 splenocytes (10^7 cells, i.v. injection). After 8–12 weeks, these mice were euthanized, T cells isolated from the spleen by non-touch T cell MACS sorting and transferred into naïve CBA mice. Induction therapy with vehicle or ABT-737 (4 injections of 50 mg/kg ABT-737 over 2 days) was started at the same day. Recipients were injected with 10^7 B6 splenocytes (DST) on day 0 and with MR1 (0.25 mg/injection, i.p.) on day 0, 3, 6, 11 and 14 after DST. On day 7 after DST, recipients received 50x 10^6 B6 bone marrow cells. Four weeks later, skin transplantation from B6 and BALB/c donors was performed. **(B)** Level of chimerism in peripheral blood was measured 14 weeks after BMT (n=7/group). Skin graft survival in the vehicle and in the ABT-737 group is reported in **(C)** and **(D)**,respectively (n=7/group). **(E)** Tolerant mice initially treated with ABT-737 were re-transplanted with BALB/c and B6 skin grafts 141 days after first transplantation. Skin graft survival after re-transplantation is shown in **(E)** (n=7/group).

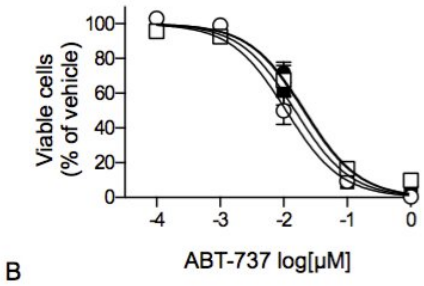
this process was not precluded by adoptive transfer of Tm. Although the intrinsic properties of memory cells do not represent a barrier for the pro-apoptotic effect of ABT-737 (Figure 2.1), our data indicate that a short conditioning therapy with ABT-737 did not induce a complete Tm depletion (Figure 2.4 H and I). It seems rather that ABT-737 induced a sufficient clone size reduction in the alloreactive Tm pool to restore sensitivity to costimulation blockade. In a clinical perspective, this is an important finding, since it supports the hypothesis that a potentially harmful complete Tm depletion is not required to overcome memory barriers in transplantation.

The mixed chimerism experiment presented in Figure 2.5 represents another important potential application of this concept. Among the different experimental strategies to induce tolerance in mice, only mixed chimerism was successfully translated into clinical protocols [250, 251]. However, a broad clinical application of this approach is currently hampered by the toxicity of the induction therapy that is required to tolerize the peripheral T cell compartment and to achieve engraftment of allogeneic bone marrow, which is necessary for the establishment of central deletional tolerance [254]. The establishment of a clinical protocol to induce mixed chimerism based on costimulation blockade would represent a major advance in the field, but is currently precluded at least in part because of the barrier provided by Tm [231]. A short induction therapy to reduce Tm by using Bcl-2 inhibitors might therefore represent the optimal solution to this central problem, particularly because of the beneficial effect of ABT-737 on mixed chimerism induction in combination with cyclosporine A [191, 243].

In consideration of phase I clinical trials with navitoclax (the orally bioavailable counterpart of ABT-737), it seems that an induction therapy with Bcl-2 inhibitors should also have an acceptable toxicity in the context of a non-malignant condition, and therefore may find an application in other indications with a critical role of Tm, such as transplantation or autoimmunity. For a clinical application our data need to be validated in human cells, but since the apoptosis pathway is highly conserved throughout evolution, similar results are likely to be obtained in large animals and humans.

Other immunological barriers typically encountered in sensitized recipients cannot be addressed using this approach: particularly, donor-specific antibodies cannot be reduced by Bcl-2 inhibitors. This aspect does not seem to play a critical role in skin graft rejec-

A



B

	IC50 μM	95% CI
CD8+CD44- (●)	0.022	0.016-0.028
CD8+CD44+ (■)	0.015	0.011-0.021
CD4+CD44- (○)	0.011	0.007-0.015
CD4+CD44+ (□)	0.020	0.014-0.029

Figure 2.6: (Supplementary) In vivo generated Tm are sensitive to ABT-737
(A, B) To assess the sensitivity of in vivo generated Tm to ABT-737, CBA mice were primed with B6 splenocytes. Eight weeks after priming, sensitivity to ABT-737 was determined by measuring cell viability after 12h incubation with ABT-737. Sensitivity to ABT-737 induced apoptosis of naïve (CD44-) and memory (CD44+) CD4 and CD8 T cells is shown. CI, confidence interval; IC50, half maximal inhibitory concentration.

tion, but it is generally considered a major problem for bone marrow transplantation in mice [255]. For this reason we were not able to induce mixed chimerim in sensitized recipients with circulating donor-specific antibodies (not shown). Notably, the same problem can be controlled more efficiently in patients using peritransplant plasmapheresis or immunoadsorption and should therefore not limit the clinical applicability of this strategy. However, studies including memory B cells are required to further develop this principle toward a clinical protocol.

In conclusion, Tm reduction by Bcl-2 inhibition might represent an important advance in the field of transplantation. Potential clinical applications are wide, from the desensitization of HLA-sensitized patients to the further development of tolerance induction protocols.

Acknowledgments

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Contribution of S.S.G. to the manuscript

Performed experiments shown in Figure 2.2 (A, B), entire Figure 2.3 (A-C) and entire Figure 2.4 (A-I) and helped writing the revised version of the manuscript.

2.2 Distinctive expression of Bcl-2 factors in regulatory T cells determines a molecular target to induce immunological tolerance

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Abstract

The molecular characterization of functionally different lymphocytes is critical for identifying novel pharmacological targets for immunotherapy. We found differences in the regulation of the intrinsic apoptosis pathway among effector and regulatory T cells. Targeted pharmacological modulation of this pathway with a small molecule Bcl-2/Bcl-xL inhibitor (ABT-737) allowed selective depletion of effector T cells and consecutive enrichment of regulatory T cells in vivo. Treatment with ABT-737 resulted in a tolerogenic milieu in peripheral lymphatic tissues, which was exploited to prevent allograft rejection in a stringent fully MHC-mismatched skin transplantation model, to induce immunological tolerance in combination with bone marrow transplantation and to alleviate graft-versus-host disease. This concept has the potential to find various applications for immunotherapy, since it allows pharmacologic exploitation of the immunomodulatory properties of regulatory T cells without the need for cell manipulation ex vivo.

Introduction

Maintenance of immunological tolerance requires functional CD4+CD25+FoxP3+ regulatory T cells (Tregs). Lack of Tregs results in fatal autoimmune lymphoproliferative disorder in mice and the IPEX syndrome in humans [108, 109]. The immunomodulatory potency of Tregs attracted great interest for immunotherapy in autoimmunity, allergy and transplantation [256]. The promising results obtained by adoptive transfer of Tregs in experimental models are currently being translated into clinical pilot studies of type 1 diabetes, graft-versus-host disease (GVHD) and solid organ transplantation [257, 258]. However, individualized cell therapy requires specific infrastructure for Treg isolation and expansion, bears high costs and potential risks regarding stability, homogeneity and fate of transferred Tregs [259, 260]. A better understanding of the physiological mechanisms determining Treg development and survival might lead to alternative strategies to exploit their immunomodulatory properties directly in vivo.

Apoptosis is instrumental in shaping the immune cell repertoire and maintaining immune homeostasis. The intrinsic apoptosis pathway comprising pro- and anti-apoptotic Bcl-2 factors is critically involved in controlling deletional tolerance in the thymus [54, 261, 262], selection of high affinity clones [240], contraction of the Teff pool after antigenic clearance [66], and maintenance of long-lived memory T cells [60]. Importantly, distinct T cell subpopulations express different levels of Bcl-2 family proteins. A direct comparison of the gene expression profile in Teffs and Tregs revealed a slightly higher expression of all anti-apoptotic factors in naïve Tregs with the most relevant difference in Bcl-xL [71].

IL-2 is pivotal in Treg biology. It promotes Treg development in the thymus [263], competitive fitness [264], and peripheral homeostasis of Treg cell populations [261, 265] . Defective IL-2 signalling in mice lacking either IL-2 production or components of the IL-2 receptor (Il-2Rα or Il-2Rβ) suffer from lymphoproliferative syndrome [264]. Furthermore, reduced IL-2 production in inflamed islets and the consequential decrease in Treg/effector T cell (Teff) ratio and Treg dysfunction results in type 1 diabetes in NOD mice [266]. Notably, IL-2 signaling was involved in the maintenance of Treg survival and homeostatic proliferation through the regulation of the anti-apoptotic Bcl-2 factor Mcl-1 [70].

We hypothesized that the differential expression of Bcl-2 factors among lymphocyte subpopulations with distinct immuno-

logical functions, might represent a novel target for immunotherapy. Particularly, small molecules Bcl-2 inhibitors selectively targeting determined Bcl-2 family members might provide a novel pharmacological tool for immunomodulation through a selective depletion of lymphocyte subsets.

Materials and Methods

Mice C57BL/6 (B6, H-2^b), CBA (H-2^k), BALB/c (H-2^d) B6xCBA F1 (H-$2^{b/k}$), B6.Cg-FoxP3tm1Mal/J (FoxP3-GFP, H-2^b) and B6-Tg(FoxP3-DTR/EGFP)$^{23.3Spar}$ (DEREG, H-2^b) mice were housed under pathogen-free conditions at the University of Zürich and used for experiments at age 8 to 16 weeks, always in age and sex matched groups. FoxP3-GFP mice express an eGFP under the control of the FoxP3 transcription factor [267] and were kindly provided by Bernard Malissen (Centre d'Immunologie de Marseille-Luminy, France). With ageing, these mice seem to express higher percentage of Tregs. For this reason, FoxP3-GFP mice were always perfectly age-matched within one experiment. DEREG mice express a FoxP3 BAC-driven primate diphtheria toxin (DT) receptor fused to eGFP, allowing to specifically depleting FoxP3+ cells with DT administration [268]. DEREG mice were purchased from The Jackson Laboratory and B6, CBA, BALB/c and B6xCBA F1 mice were purchased from Harlan Laboratories. All animal experiments were performed according to protocols approved by the local legal authority (Veterinary office, Canton of Zürich, Switzerland).

Fluorescent activated cell sorting (FACS) FACS analyses were performed with a FACS Canto II or LSR Fortessa II (Becton Dickinson). The following antibodies were used to stain 1x 10^6 cells of single cell suspensions of spleen or peripheral blood: CD4 (GK1.5) -PE, -APC, -AF700, CD8 (eBioH35.-17.2) -APC, B220 (RA3-6B2) -PE, CD25 (PC61.5) -PE/Cy7, -APC, F(ab')2-FITC anti-rabbit IgG, FoxP3 (FJK-16s) -biotin, streptavidin-APC (all eBioscience), H-2Kk (AF3-12.1.3) -FITC, CD8 (53-6.7) -APC/Cy7 (all BioLegend), Bcl-2 (3F11) -FITC (Becton Dickinson), Mcl-1 (Y37), Bcl-2A1 (EP517Y) (both Abcam), Bcl-xL (54H6) -Alexa488, Bim (polyclonal) (both Cell Signaling Technologies). For live-dead discrimination PI, 7-AAD or Zombie Aqua fixable viability kit (BioLegend) were used. Mice were bled sublingual and chimersim was analyzed in peripheral leukocytes at different time points after BM transplantation, and donor-derived cells were identified by staining of donor H-$2K^k$. From the signal measured in a positive control (CBA) the background signal

of a negative control (B6) was subtracted and converted to be 100%. The same formula was then used to calculate the percentage of donor-derived cells in mixed chimeras. For intracellular stainings, a FoxP3 staining buffer set (eBioscience) was used according to the manufacturer's instructions.

ABT-737 sensitivity in vitro

For in vitro experiments, ABT-737 (AbbVie Bioresearch) and sabutoclax (Bl-97C1, AdooQ Bioscience) were dissolved in DMSO at a concentration of 5 mM and then further diluted in RPMI medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 ug/ml streptomycin, 50 µM 2-mercaptoethanol. Either freshly isolated, in vitro IL-2 (RnD Systems, PeproTech) stimulated (3 days with 100 U/ml) or alloantigen stimulated FoxP3-GFP splenocytes (see below) were plated in 96-well plates in culture medium at a concentration of 2.5x 10^6 cells/ml. Different amounts of ABT-737/sabutoclax or DMSO vehicle control were added to cells and incubated for 12 h at 37 degrees and 5% CO^2. Cells were washed 3x, stained, and viability of CD4+(CD25+)GFP+ and CD4+(CD25-)GFP- cell populations was assessed by PI or 7-AAD exclusion. Absolute cell counts per well were determined by the addition of absolute counting beads (Invitrogen). Surviving cell numbers of each population were normalized to the corresponding vehicle condition.

iTreg generation

For iTreg generation, mixed lymphocyte reactions were performed in 24-well plates. CBA splenocytes (stimulators) were depleted of T cells (CD3 microbeads) and FoxP3-GFP splenocytes (responders) were depleted of CD25+ cells by automatic magnetic cell separation according to manufacturer's instructions (Miltenyi Biotec) and then plated at a 1:1 ratio at a concentration of 4x 10^6 cells/ml. Anti-CD154 antibody (50 µg/ml, MR1, Bio-X-cell) was added and cells were incubated at 37 degrees and 5% CO^2 . After 24 h of stimulation, 5 ng/ml TGF-β (RnD Systems, PeproTech) and 100 U/ml IL-2 were added to the cultures. After another 72 h of stimulation, all cells were pooled and plated in 96-well plates in order to test ABT-737 sensitivity (see above).

In vivo antibody and drug treatments

Antibodies against CD154 (MR1), CD25 (PC61.5) (Bio-X-cell) and GITR (DTA-1, provided by S. Sakaguchi [269]) were diluted in PBS and injected i.p. as described below. Diphtheria toxin (DT, 1 µg, Calbiochem) was administered by i.p. injection on two consecutive days [270]. For in vivo application, ABT-737 was dissolved in polyethylene gly-

col, Tween 80, DMSO and dextrose solution and injected i.p. at 50 mg/kg. CsA (Enzo Life Sciences) was dissolved in a Cremaphor EL/ethanol solution, then diluted in HBSS and injected s.c. at 10 mg/kg. Control mice were treated with the corresponding vehicles.

Short- and long-term ABT-737 treatment
For short term ABT-737 treatment, mice received 5 ABT-737 injections from day -3 to day -1, before their spleens were harvested and analysed on day 0. For longterm ABT-737 treatment, mice received injections for four weeks every other day and were bled sublingual on day 0, day 7, day 14, day 21 and day 28.

Skin grafting
In all in vivo skin graft experiments, B6 or DEREG mice were recipients, CBA mice were donors and BALB/c mice were 3rd party controls. CBA and BALB/c are fully MHC-mismatched to B6/ DEREG and among each other. For skin transplantations, recipient mice were anesthetized with ketamine/xylazine, pain treated with carprofen (NSAID) and shaved. Full thickness tail skin (about 1 cm^2) of donors and 3rd party controls was grafted to the back and considered rejected, when $<10\%$ of the graft remained viable (non-blinded).

Donor-specific transfusion (DST)
In the DST experiment, recipient mice were treated with a total of 5 injections of ABT-737/vehicle on days -3 to -1, and anti-CD154 antibodies on day 0, 6, 11 and 14 (0.25 mg each). On day 0 recipients received 1x 10^7 donor splenocytes in Media 199 containing 10 mM HEPES, 10 µg/ml DNAse and 4 µg/ml Gentamycin by tail vein injection. B6 recipients received anti-GITR (4 mg) antibodies on day 6 and DEREG recipients DT on day 6/ 7 and skin was grafted on day 7.

Bone marrow transplantation (BMT)
The ABT-737 tolerance protocol was described in detail previously [191]. The conditioning of mice consisted of ABT-737 and CsA treatment with a total of 5 injections on days -3 to -1, and 1 daily injection until day 11. Anti-CD154 antibodies (2 mg) were administered once 6 hours before the transplantation of 25x 10^6 donor BM cells by tail vein injection on day 0 (in the same medium as described above for DST). Tregs were depleted with anti-CD25 antibodies (1.25 mg) or blocked with anti-GITR antibodies (4 mg) on day -1. Peripheral blood chimerism was monitored and 8 weeks after BMT all mice received skin grafts.

Graft-versus-host disease (GVHD)
On day 0 B6xCBA F1 recipient mice were lethally irradiated (900cGy) with a Cs source. 6h later, mice were transplanted with 10 mio DEREG BM cells and 20 mio

DEREG splenocytes by tail vein injection (in the same medium as described above for DST), which corresponded to 5.7 mio T cells in total. Furthermore, mice received cefazolin (antibiotic, 1 mg) after transplantation on day 0 and on day 2. ABT-737 and/or CsA treatment started on day-1 and was continued until day 10, and following doses were injected; day -1: 75 mg/kg ABT-737 and 15 mg/kg CsA, day 0: 100 mg/kg ABT-737 and 15 mg/kg CsA, day 1 to day 3: 50 mg/kg ABT-737 and 10 mg/kg CsA, day 4 to day 10: 25-50 mg/kg ABT-737 (mostly 35 mg/kg) and 10 mg/ml CsA. Thereafter, mice continued to receive 10 mg/kg CsA until death. In the initial phase after irradiation and T cell transfer, some of the already weakened animals did not well tolerate ABT-737 treatment and about 25% of animals died during the first few days after transplantation. Accordingly, ABT-737 dose was adjusted to the condition of individual animals. Tregs were depleted on day 14/15 by DT administration. Mice were monitored daily and severity of GVHD was assessed non-blinded by a score consisting of (a) posture/activity, (b) fur texture, (c) skin integrity, (d) body weight loss, as previously described in [271].

In vivo MLR

For the „in vivo MLR“ experiment, F1 (B6xCBA) recipient mice received anti-CD154 antibodies (2 mg) 6 hours before tail vein injection of 30x 106 B6.FoxP3-GFP splenocytes (in the medium as described above for DST). The following 3 days, mice received 1 daily injection of ABT-737/vehicle, and CsA. Spleens were harvested and analyzed on day 4.

Hemisplenectomy

For hemisplenectomy, mice where anesthetized by isoflurane inhalation, pain treated with buprenorphine and treated with cefazolin (antibiotic). The abdomen was opened by midline incision, and two ligations were placed around the inferior vascular pedical and the middle of the spleen, before half of the spleen was removed inferior to the intrasplenic ligation. The abdominal wall was closed with continuous suture.

Statistics

Statistical analysis was performed with GraphPad Prism software. Unpaired two-tailed Student's t-test was used to assess statistical differences between groups. IC50 values were calculated using a log(inhibitor) vs. (normalized) response model (variable slope) for in vitro ABT-737 and sabutoclax sensitivity analysis. Survival in Kaplan-Meier survival curves was compared using Log-Rank test. In animal experiments, each data point represents results from one single mouse, whereas in in vitro experiments, one

data point represents the mean of technical replicates (usually triplicates). $P<0.05$ was considered significant, $*P<0.05$; $**P<0.01$; $***P<0.001$, n.s., not significant.

Results

Distinctive expression of anti-apoptotic Bcl-2 factors in T cell subtypes

We compared the protein expression pattern of Bcl-2 factors in naïve Tregs (CD4+CD25+ FoxP3+) vs. Teffs (CD4+CD25-FoxP3-) and upon stimulation with IL-2. Unstimulated cells displayed similar expression levels of anti-apoptotic Bcl-2 factors with the exception of Bcl-xL, of which the previously reported higher gene expression in Tregs [71] was confirmed on the protein level (Figure 2.7 A). In contrast, IL-2 stimulation for three days dramatically changed the expression of Bcl-2 family proteins. Tregs displayed higher expression of Bcl-xL and Bcl-2, and to a smaller extent of Mcl-1 and Bcl-2A1 (A1) (Figure 2.7 B). This distinctive molecular phenotype among Tregs and Teffs was exploited as pharmacological target for Treg enrichment.

Enrichment of Tregs by Bcl-2/Bcl-xL inhibition in vitro and in vivo

The small molecule ABT-737 binds Bcl-2, Bcl-xL and Bcl-w proteins with high affinity, and Mcl-1 and A1 with much lower affinity (Figure 2.7 C) [97]. Thus, cell populations whose survival depends primarily on anti-apoptotic Mcl-1 and A1 are resistant to ABT-737-mediated apoptosis [61, 272]. Moreover, although ABT-737 binds Bcl-2 and Bcl-xL with similar affinity, increased Bcl-xL expression in lymphocytes was shown to confer resistance to ABT-737-mediated killing [273]. Based on the higher Bcl-xL levels in naïve and activated Tregs and the predominant importance of Mcl-1 in Treg survival [70, 71], we hypothesized that the Treg compartment would be protected from the pro-apoptotic effect of ABT-737. For quantitative analysis in vitro, we assessed cell viability after overnight incubation of freshly isolated splenocytes with ABT-737. Tregs displayed a consistent survival advantage over Teffs as reflected in a 13.2x higher IC50 value (Figure 2.7 D). As a result, ABT-737 treatment led to a relative enrichment of Tregs in vitro (from 14.5% to 29.7% Tregs among CD4+ cells with increasing ABT-737 concentrations, Figure 2.7 D). This effect was even more pronounced after IL-2 stimulation. After a three-day IL-2 incubation, Tregs were extremely resistant to ABT-737 compared to Teffs (approx. 3300x higher IC50 value), leading to a further Treg enrichment in vitro (up to 47%, Figure 2.7 F, G). Resistance to Bcl-2 inhibition in IL-2 stimulated Tregs was

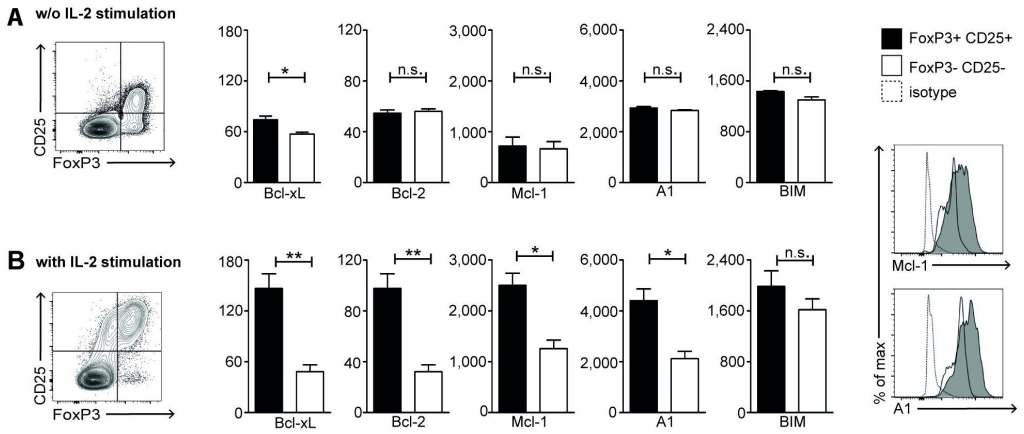

Figure 2.7: continued on next page

markedly reduced when ABT-737 was replaced by the pan-Bcl-2 inhibitor subatoclax, which binds also Mcl-1 and A1 with high affinity (Figure 2.7 C, H, I) [96]. This finding is well illustrated by comparing IC50 values of Tregs and Teffs under these different conditions: Resistance of Tregs in comparison to Teffs under ABT-737 treatment increased several magnitudes upon IL-2 stimulation, while sabutoclax resistance increased only slightly (Figure 2.7 J). This indicates that the differential expression of Bcl-2 family proteins among Tregs and Teffs, and particularly the up-regulation of Mcl-1 and A1 upon IL-2 stimulation, was responsible for resistance to ABT-737-mediated killing.

This intrinsic property of Tregs was confirmed in vivo. Naïve mice were treated for three days with a total of five injections of ABT-737. This resulted in moderate lymphopenia and in a reduced number of CD4+ cells in the spleen (Figure 2.8 A) [241, 243]. However, mice treated with ABT-737 had a significantly higher percentage of Tregs among CD4+ cells in comparison to vehicle controls (Figure 2.8 B, C). Hemisplenectomy allowed analyzing splenocytes at two different time points in the same mouse, and it showed that this effect was temporary; ten days after stopping ABT-737 treatment, the Treg/Teff balance was re-established to a similar level as in vehicle-treated animals (Figure 2.8 D). Furthermore, prolonged treatment with ABT-737 resulted in a transient Treg enrichment with a peak after two weeks (Figure 2.8 E). Thus, Bcl-2/Bcl-xL inhibition resulted in a relative temporary enrichment of Tregs in vitro and in vivo.

Antigen-stimulated Tregs are resistant to

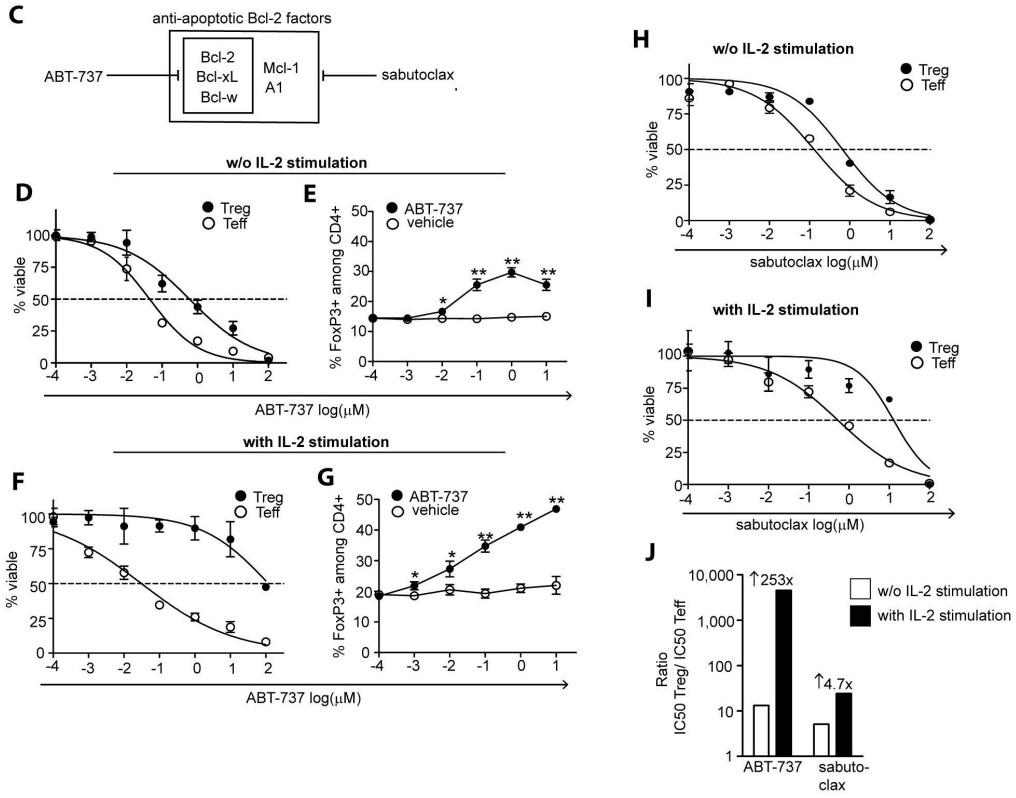

Figure 2.7: Differential expression of Bcl-2 factors among Tregs and Teffs mediates resistance of Tregs to ABT-737 in vitro.
(A, D, E, H) FoxP3-GFP splenocytes without stimulation or **(B, F, G, I)** upon 3 days IL-2 stimulation were used for in vitro experiments. **(A, B)** Representative FACS plots showing CD25 and FoxP3 expression in viable CD4+ cells. Quantification of median expression of Bcl-xL, Bcl-2, Mcl-1, A1 and Bim in CD4+CD25+FoxP3+ cells (black) and CD4+CD25-FoxP3- cells (white). The stainings were performed in triplicates and repeated three times, one representative experiment is shown. FACS plots showing Mcl-1 and A1 expression in Tregs (grey) and Teffs (white) upon IL-2 stimulation. **(C)** Binding profiles of ABT-737 and sabutoclax. **(D, F, H, I)** Cells were incubated for 12h with the indicated concentrations of ABT-737 or sabutoclax, respectively. The number of viable CD4+FoxP3+ (black) and CD4+FoxP3- (white) cells per well was assessed by FACS and normalized to corresponding vehicle controls. Curve represents a log(inhibitor) vs. normalized response curve. **(E, G)** The percentage of FoxP3+ cells among CD4+ cells was assessed after 12h ABT-737/vehicle incubation. In vitro titration experiments were performed 2 to 4 times, representative experiments are shown. **(J)** Illustration of the ratio of IC50 of Tregs over IC50 of Teffs in naïve cells (white) and after IL-2 stimulation (black). All data represent mean +/- s.e.m., *P<0.05, **P<0.01, t-test was used to assess differences between cell populations **(A, B)** and treatment groups at certain concentrations **(E, G)**

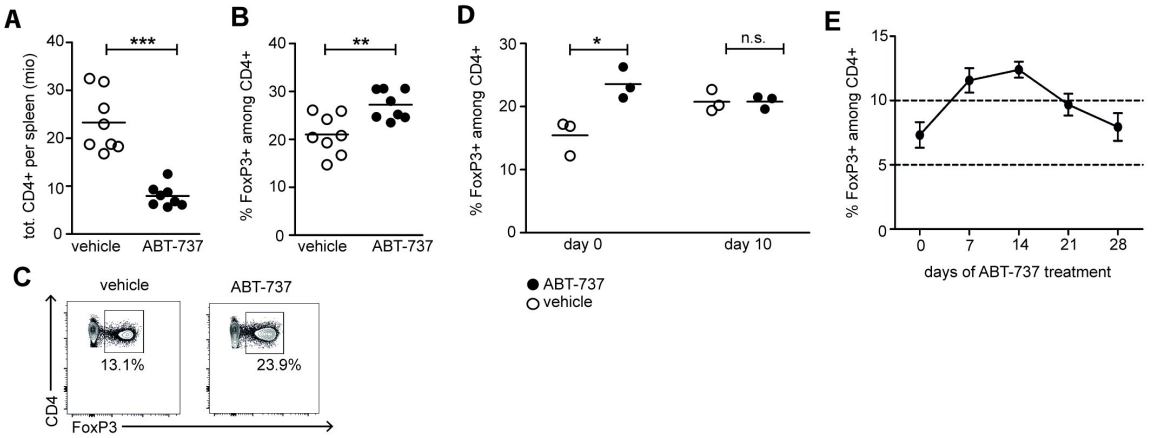

Figure 2.8: ABT-737 resistance of Tregs in vivo.
(A-D) FoxP3-GFP mice were treated with vehicle or ABT-737 i.p. for 3 days prior to harvesting. **(A)** Total counts of CD4+ cells per spleen and **(B)** the percentage of FoxP3+ cells among CD4+ cells. Pooled data of two out of four independent experiments, n = 8/group. **(C)** Representative FACS plots of viable CD4+ splenocytes in vehicle and ABT-737 treated animals. **(D)** Percentage of FoxP3+ cells among CD4+ cells in half spleen, n = 3/group. **(E)** Percentage of FoxP3+ cells among peripheral blood CD4+ cells during four-week ABT-737 treatment, n = 4. All data represent mean +/- s.e.m., *P<0.05, **P<0.01, ***P<0.001. Differences between treatment groups were assessed with t-test.

ABT-737-mediated apoptosis

In contrast to naturally occurring thymus-derived Tregs (nTregs), peripherally-derived Tregs (pTregs) convert in peripheral lymphoid tissues from naïve CD4+FoxP3-CD25- T cells. Minimal requirements for pTreg generation are antigenic stimulation, TGF-β and IL-2 [274–276]. Inhibition of the CD40-CD154 costimulatory pathway further favours the induction of Tregs [277, 278]. Similar stimuli allow the generation of in vitro-induced Tregs (iTregs). To investigate the impact of selective Bcl-2/Bcl-xL inhibition on survival of iTregs, we induced Tregs from CD25-depleted naïve splenocytes by allogeneic stimulation under costimulation blockade (anti-CD154, MR1) and TGF-β/IL-2 cytokines. ABT-737 incubation revealed high resistance of alloantigen-activated iTregs, similar to the previously observed resistance of activated CD25+ Teffs (IC50 value 36'000x higher than in CD25- Teffs, Figure 2.9 A) [61]. To investigate the properties of pTregs, we took advantage of an „in vivo mixed-lymphocyte reaction model". B6.FoxP3-GFP splenocytes were transferred into F1 (B6xCBA) recipients under costimulation blockade with anti-CD154 antibodies, followed by ABT-737 or vehicle treatment for three days. Low-dose cyclosporine

A (CsA) was combined with ABT-737 to prevent T cell receptor-calcineurin-NFAT1-dependent upregulation of A1 in Teffs, and thus preventing resistance of Teffs upon antigenic stimulation [61]. ABT-737 treatment resulted in moderate lymphopenia and in a marked enrichment of donor-derived Tregs among transferred cells (Figure 2.9 B). Taken together, antigen-stimulated iTregs and pTregs were resistant to Bcl-2/Bcl-xL inhibition, and exposure to ABT-737 resulted in a shift of the balance between Tregs and Teffs in favour of the Treg compartment.

Treg enrichment by ABT-737 inhibits GVHD

Tregs are recognized for alleviating the course of GVHD, and Treg therapy has been successfully tested in this condition [258]. Therefore, as first functional application of our concept, we aimed to investigate whether ABT-737 mediated Treg enrichment has a favourable impact on GVHD in a classical model. Recipient F1 (B6xCBA) mice were lethally irradiated and reconstituted with allogenic B6.DEREG bone marrow and T cells. DEREG mice express a FoxP3-driven primate diphtheria toxin (DT) receptor, which allows selective Treg depletion by DT administration [268]. Body weight and a GVHD score consisting of activity/posture, fur texture, skin integrity and weight loss was assessed daily. Mice were either treated with combined ABT-737 and low-dose CsA or CsA alone, starting one day before cell transfer and continued for ten days (Figure 2.9 C). GVHD onset was about ten days after transplantation in control mice treated only with CsA, and the disease progressed to lethality within three weeks (Figure 2.9 D-F). ABT-737 and CsA treated animals presented a markedly attenuated course of GVHD. Importantly, Tregs were integral in mediating this positive effect of treatment, as Treg depletion at day 14 after transplantation induced a rapid onset of GVHD (median survival time (MST) in ABT-737 + CsA treated group 49.5 days vs. 25.5 days in Treg depleted group, $p = 0.0006$). None of the ABT-737 treated animals developed skin lesions, and thus they did not reach the maximal GVHD score. In conclusion, Tregs enrichment in vivo resulted in an important attenuation of GVHD across full MHC barriers.

Treg enrichment by ABT-737 induces donor-specific hyporesponsiveness

To investigate whether this immunomodulatory effect was antigen-specific, we took advantage of an established model of full MHC-mismatched skin transplantation after donor-specific transfusion (DST) under costimulation blockade [132]. B6 or B6.DEREG recipient mice received donor-type spleno-

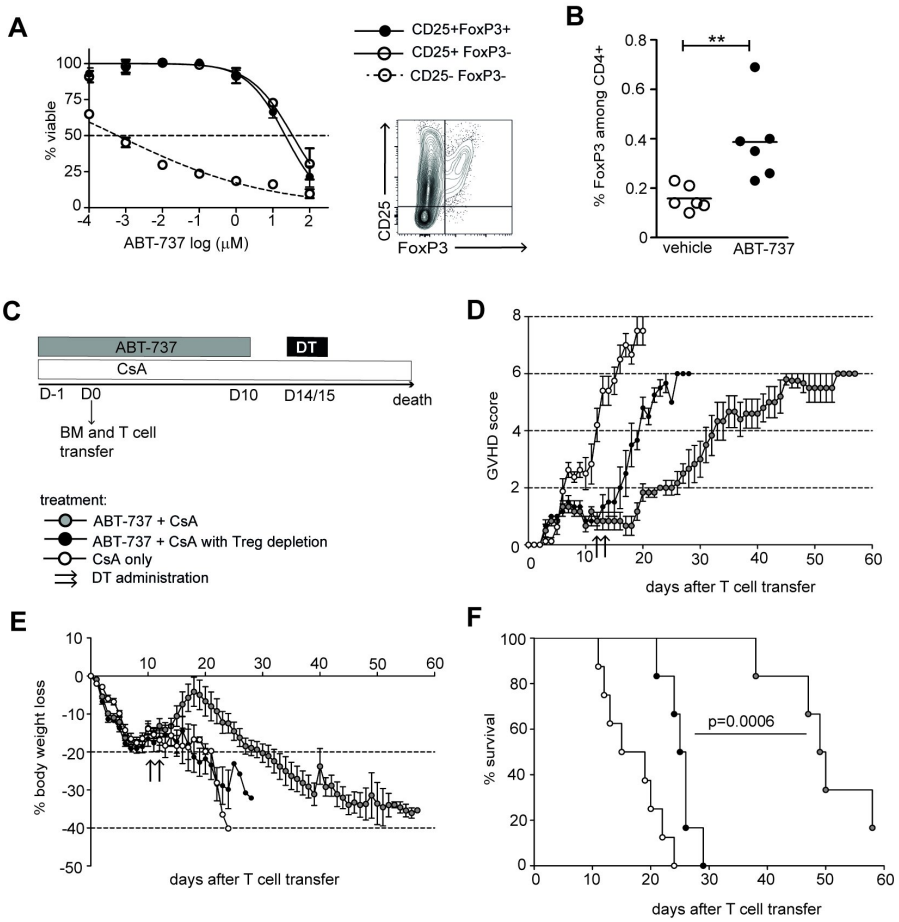

Figure 2.9: Alloantigen-activated Tregs are resistant to ABT-737-mediated apoptosis and alleviate GVHD.
(A) iTregs were generated in an mixed-lymphocyte reaction with CD25-depleted B6.FoxP3-GFP responders, CD3-depleted CBA stimulators and anti-CD154 antibodies for 24h, and additional IL-2/TGF-β for 3 days. After 12h incubation with ABT-737 or vehicle, viable cell counts were assessed by FACS and normalized to corresponding vehicle controls. Curve represents a log(inhibitor) vs. response curve. Representative FACS plot of iTregs. One of two independent experiments is shown **(B)** B6.FoxP3-GFP splenocytes were transferred to F1 (B6xCBA) recipients under anti-CD154 costimulation blockade and treated with ABT-737 or vehicle, and CsA for 3 days. Spleens were analyzed on day 4 by FACS. The ratio between the absolute number of FoxP3+ and FoxP3- cells per spleen among transferred cells (H-2Kk-) is shown. Pooled data of two independent experiments, n = 6/group. **(C)** Experimental setup for GVHD experiment: F1 (B6xCBA) mice received DEREG bone marrow cells and T cells after lethal irradiation on day 0 and treatment from day -1 until day 10 with ABT-737, and/or CsA until death. Tregs were depleted by DT administration on day 14/15. **(D)** GVHD score, **(E)** body weight loss and **(F)** survival, n = 6-8/group. All data represent mean +/- s.e.m., **P<0.01. Differences between treatment groups was assessed with t-test **(B)** and log-rank test **(F)**.

cytes (CBA) under the effect of anti-CD154 antibody (Figure 2.10 A). This induction therapy leads to deletion/anergy of donor-specific Teffs, favours the generation of Tregs and results in transient donor-specific hyporesponsiveness [279]. We combined this protocol with a three-day ABT-737 treatment prior to i.v. splenocyte injection. Seven days after DST, recipient mice received donor-type (CBA) and 3rd party control (BALB/c) skin grafts. In this stringent strain combination, the original DST protocol (vehicle group) did not significantly prolong skin graft survival (Fig. 4B). However, the short additional treatment with ABT-737 was sufficient to markedly prolong donor-type skin graft survival up to more than 100 days in some recipients (MST 39/87 days for ABT-737 and 12 days for vehicle, Figure 2.10 B, D). Notably, this effect was donor-specific and not due to generalized immunosuppression, as demonstrated by the only slightly delayed rejection of 3rd party grafts. To assess the importance of Tregs in ABT-737-induced donor-specific hyporesponsiveness in this model, we depleted Tregs by DT injection in DEREG recipients or inhibited their function by GITR (glucocorticoid-induced TNFR family related gene) ligation the day prior to skin transplantation [280]. In absence of a functional Treg compartment, all skin grafts were rapidly rejected, and no difference between donor-type and 3rd party grafts was observed (Figure 2.10 C, E). Thus, although ABT-737 might have additional Treg-independent effects influencing graft survival, we show here that the ABT-737-induced hyporesponsiveness was critically dependent on Tregs, and the transient alteration of the Treg/Teff balance after ABT-737 treatment was sufficient to create a strong tolerogenic milieu.

ABT-737-mediated Treg enrichment allows induction of transplantation tolerance

A transient state of Treg-mediated donor-specific hyporesponsiveness might represent an ideal environment to promote engraftment of allogeneic bone marrow cells. We previously showed that a two-week conditioning treatment with ABT-737, low-dose CsA, costimulation blockade (anti-CD154) and transplantation of 25x 10^6 bone marrow cells resulted in stable mixed lympho-hematopoietic chimerism and complete deletion of donor-reactive CD8+ cells in a fully MHC-mismatched strain combination (Figure 2.11 A) [191]. Grafting of donor-type (CBA) and 3rd party control (BALB/c) skin grafts to B6 recipients eight weeks later confirmed stable donor-specific immunological tolerance in most animals (Figure 2.11 B). We hypothesized that ABT-737 mediated

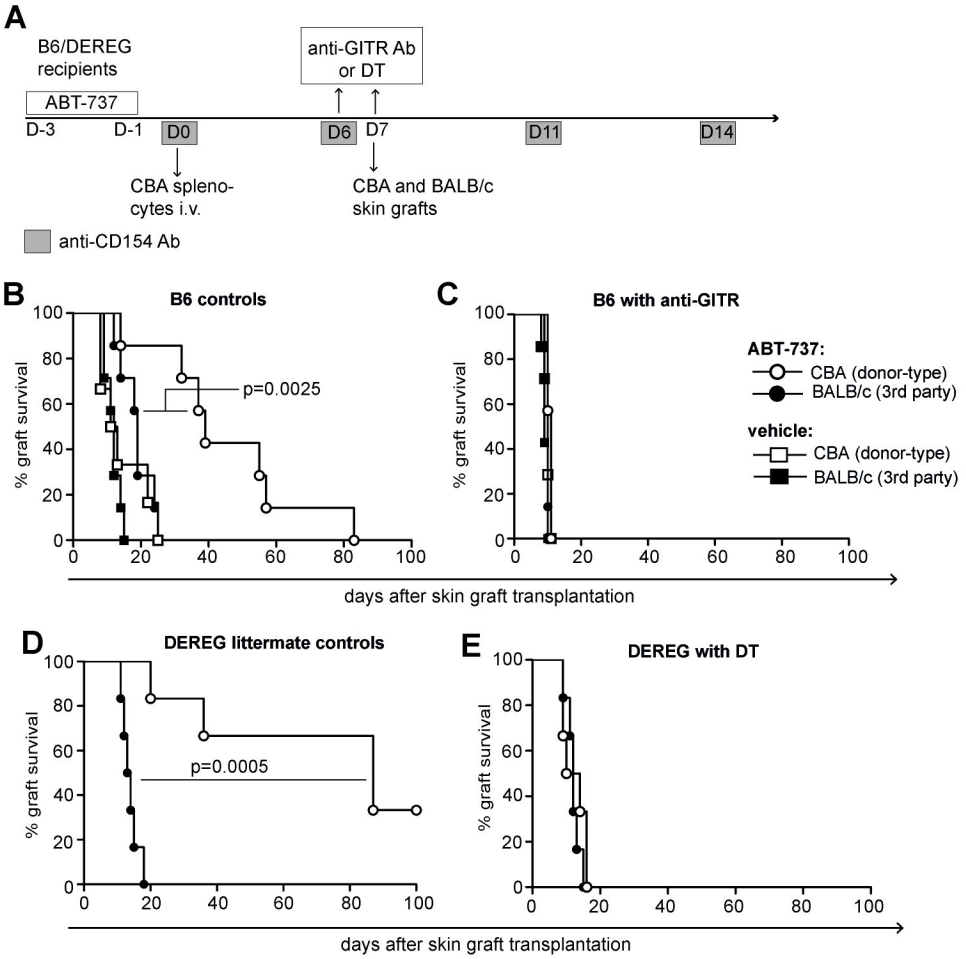

Figure 2.10: ABT-737 induces Treg-mediated donor-specific hyporesponsiveness.
(A) Experimental setup for DST experiment: B6 or DEREG mice were treated with ABT-737 or vehicle for 3 days prior transfer of CBA splenocytes on day 0 and anti-CD154 treatment. On day 7, mice received CBA (donor-type) and BALB/c (3rd party) skin grafts, and graft survival was monitored until rejection. **(B, D)** Survival of CBA and BALB/c grafts after ABT-737 and vehicle treatment **(B)** or ABT-737 treatment **(D)**. **(C)** Tregs were blocked with anti-GITR antibodies (day -1, B6 recipients) or **(E)** by DT injection (day -1 and 0, DEREG recipients). n = 7/group, differences between treatment groups were assessed with log-rank test **(B, D)**.

Treg enrichment is a critical factor for tolerance induction in this myelosuppression-free protocol. To test this hypothesis, Tregs were either depleted with anti-CD25 antibody or functionally inhibited by GITR ligation the day prior to bone marrow transplantation. This antibody treatment indeed precluded the establishment of tolerance to skin grafts in all animals, because mixed chimerism induction was hampered in the absence of a functional Treg compartment (Figure 2.11 C, D). Thus, the transient tolerogenic milieu caused by Treg enrichment through Bcl-2/Bcl-xL inhibition was required to induce mixed chimerism without myelosuppressive conditioning.

Discussion

A dysregulated balance between regulatory and effector cell populations is often involved in overshooting immune activation, such as in transplant rejection, autoimmunity or allergies. Thus, the achievement of functional dominance of Tregs over Teffs is a major aim in a wide range of clinical conditions. Treg therapy has proven successful in various experimental models and in human pilot studies [257–260]. Large scale ex vivo generation or expansion of Tregs is feasible, but related to logistical and financial hurdles that may preclude wide-spread clinical application. An alternative strategy to manipulate the Treg/Teff balance and to achieve Treg-mediated tolerance, is the selective reduction or functional inhibition of Teffs in vivo while preserving the Treg population. To achieve this goal, pathways that are differentially regulated among these two cell populations need to be identified and pharmacologically manipulated. For example, cellular metabolism reflected in differential mTOR activity has been proposed to be such a pathway [281], and low-dose IL-2 administration selectively favoured expansion of Tregs [282].

In the present study, we identified the intrinsic apoptosis pathway as promising novel target to exploit the immunomodulatory potency of Tregs. Although there are no single anti-apoptotic factors with a non-redundant function in defined lymphocyte populations, it is generally presumed that the relative importance of apoptotic factors varies during T cell development and among different subpopulations [24]. According to previous studies, Tregs have a differential gene expression pattern of apoptotic factors compared to their effector counterparts with the biggest difference in Bcl-xL expression, which might account for differences in sensitivity to cytotoxic stress [71]. Importantly on a functional level, anti-apoptotic Mcl-1 was the predominant survival factor in Tregs, and loss of

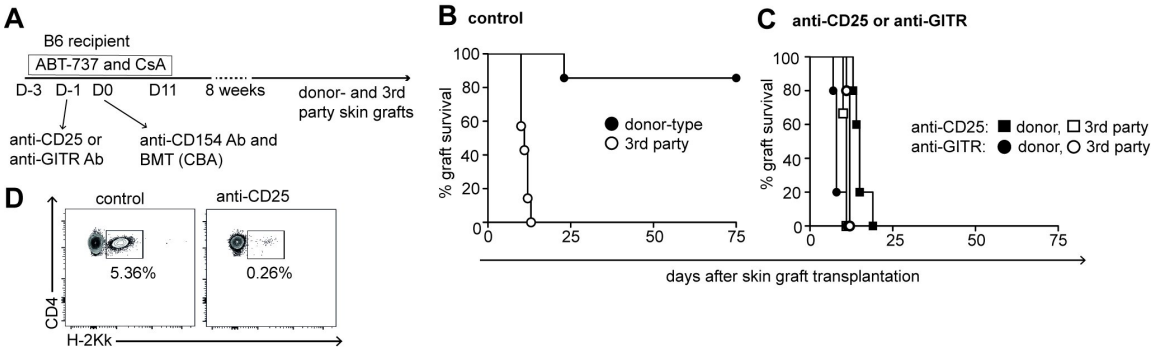

Figure 2.11: ABT-737 mediated Treg enrichment allows tolerance induction.
(A) Experimental setup of mixed chimerism tolerance protocol: B6 recipients were treated with five injections of ABT-737 and CsA during the 3 days prior transplantation of CBA bone marrow cells on day 0. Anti-CD154 antibodies were administered 6h before bone marrow transplantation, and ABT-737/CsA treatment was continued until day 11 after transplantation. Tregs were blocked by anti-GITR antibodies or depleted by anti-CD25 antibody administration at day -1. Eight weeks after transplantation, mice received CBA (donor-type) and BALB/c (3rd party) skin grafts and survival of grafts was monitored until rejection. **(B)** Graft survival of control group and **(C)** of Treg blocked/depleted groups, n = 7/group. **(D)** Representative FACS plots of peripheral CD4+ cell chimerism in control mouse and Treg depleted mouse two months after bone marrow transplantation.

Mcl-1 resulted in drastic reduction of Treg numbers. In contrast, loss of Bcl-2 did not result in a relative reduction of Tregs [70]. Thus, the relative importance of Mcl-1 and Bcl-2 varies among Tregs and Teffs. In this study, we exploited these findings to establish a novel pharmacological target for immunomodulation. Combined inhibition of the anti-apoptotic Bcl-2 and Bcl-xL induced a functionally important Treg enrichment in vivo. This concept was tested in very stringent immunological models: short-term Bcl-2/Bcl-xL inhibition was sufficient to inhibit GVHD, to inhibit allogeneic immune responses against fully MHC-mismatched skin grafts and to allow bone marrow engraftment without myelosuppression in a mixed chimersm model across full MHC barriers. Importantly, iTregs were even more resistant to ABT-737 than nTregs, and these iTregs are presumably responsible for the donor-specificity observed in skin graft experiments. This fact offers the opportunity to generate an antigen-specific tolerogenic environment with broad clinical implication for immunomodulatory therapies. In transplantation medicine, donor-specific immunosuppression represents an important step towards the prevention of rejection without the risks for infections and cancers related to non-selective immunosuppression. Evasion of apoptosis is one of the hallmarks of cancer,

as Bcl-2 factors are often overexpressed in malignant cells. Thus, large efforts are taken for developing highly selective Bcl-2 family inhibitors. To date, little is known about apoptosis regulation in human T cell subsets. For clinical translation of the proposed immunomodulatory concept, closer research on human cells will be needed, but tailored Bcl-2 inhibition is likely to translate into clinical therapies. Several Bcl-2 inhibitors have already entered clinical trials for various cancers and recently, a phase I study with ABT-199 in systemic lupus erythematosus patients has been completed [98, 283]. Bcl-2 inhibitors showed a very favourable toxicity profile so far. The present study might stimulate further research on the effect of these inhibitors on T cells subsets directly in patients and importantly, it might draw attention to the tumor immunology point-of-view when using these compounds in cancer therapy.

Acknowledgements

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Contribution of S.S.G. to the manuscript

Designed experiments together with T.F. and P.E.C. and performed all experiments except hemisplenectomy, prepared all figures and wrote the manuscript with help of T.F. and P.E.C.

2.3 Bcl-2/Bcl-xL inhibition promotes irradiation-free engraftment of HSCs - tracking down the mechanisms

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Status of the manuscript: ongoing project

Abstract

Induction therapy with a Bcl-2/Bcl-xL blocking small-molecule drug in combination with low-dose cyclosporine A and one injection of costimulation-blocking antibodies allowed the establishment of mixed chimerism in mice. The finding that such a minimal myelosuppression-free recipient conditioning was sufficient to prevent rejection of transplanted allogeneic stem cells, while promoting the engraftment of these cells in the host's bone marrow, is intriguing. In the present study, we are investigating the effects of both, Bcl-2/Bcl-xL and calcineurin inhibition, in syngeneic bone marrow transplantation. We are studying the direct effect of drug treatment on hematopoietic stem cells and the progenitor compartment, and we are testing different hypotheses that might explain the efficient engraftment of transplanted stem cells. Better understanding of the mechanisms that promote stem cell engraftment may be important for the development of novel low-toxicity conditioning protocols for bone marrow recipients.

Introduction

The transplantation of bone marrow (BM) stem cells finds application in numerous, potentially life-threatening conditions. Myelotoxic doses of irradiation and chemotherapy are needed for the eradication of hematological malignancies, and the ablated hematopoietic system needs to be reconstituted with transplanted hematopoietic stem cells (HSCs), but also inborn defects in blood cells can be corrected by BM transplantation. With the advent of reduced-intensity preparatory regimen of BM recipients, novel indications for BM transplantation could be envisaged, such as the induction of transplantation tolerance that has been successfully introduced in clinical pilot studies [194]. As hematopoietic lineage-derived cells are also involved in tissue homeostasis, for example in the kidneys, the potential of BM transplantation for improvement of non-hematologic inborn diseases may be even bigger [284, 285]. However, the risks associated with BM transplantation currently circumvent clinical translation of such experimental approaches.

HSCs stand at the apex of hematopoiesis and replenish immune cells, erythrocytes and platelets that are lost due to constant turnover. In order to fulfil their task, HSCs undergo asymmetric cell division. They self-renew to sustain the stem cell pool, and give rise to lineage-restricted, quickly cycling progenitors that terminally differentiate into the various hematopoietic lineages. As such, HSCs are integral for the maintenance of homeostasis in steady state and in response to situations that demand increased blood cell production [286]. Different stem cell populations can be discriminated based on their proliferative behaviour and self-renewal potential. Long-term HSCs (LT-HSCs) are mostly quiescent, while short-term HSCs (ST-HSCs) proliferate much more. LT-HSCs that additionally express CD150 (SLAM) have been suggested to sit at the uppermost part of the stem cell hierarchy, possessing the greatest self-renewal potential [287].

HSCs reside in a highly specialized microenvironment called the „stem cell niche“, which consists of cellular and molecular components that regulate stem cell function by complex interactions. Both, the cells of the niche and the HSCs are well-equipped to integrate signals from the periphery, and stem cell behaviour is adopted accordingly. The thrombopoietin (TPO) feedback-loop exemplifies this concept. TPO is constitutively expressed in the liver, and it is cleared from the peripheral circulation by platelets that express the TPO receptor c-mpl [288, 289]. If the platelet mass decreases, for example

due to bleeding, the TPO level rises. As HSCs, early progenitors of all lineages and megakaryocytes also express c-mpl, the rising TPO levels stimulate megakaryopoiesis and platelet production directly in the BM compartment. Further, TPO is needed for the maintenance of HSCs in steady state, as TPO KO and c-mpl KO mice both display largely reduced HSC numbers [290]. Interestingly, LT-HSCs have been shown to express higher levels of c-mpl than ST-HSCs and multipotent progenitors (MPPs) [291].

The general perception is that stem cell niches need to be opened at least partially prior transplantation, in order to create „space“ for the transplanted stem cells. This is often achieved by irradiation of the recipient. It has been shown that approximately 1-5% of HSCs leave their niche each day to enter peripheral circulation [292]. Thus, a small number of niches is constantly unoccupied, which potentially can absorb transplanted syngeneic HSCs present in the circulation in unconditioned hosts. This explains why repeated infusion of BM cells resulted in higher chimerism than one single bolus of a higher cell dose [292, 293]. However, a mega dose of 200 mio allogenic BM cells under costimulation blockade equally allowed to achieve chimerism [188]. The mechanisms of how such a mega dose can result in a high engraftment level is not understood yet. Maybe the vast number of transplanted HSCs displaced endogenous HSCs from their niche, or the HSCs persisted for some time in the blood and continuously filled freshly opened niches.

In a recently published article, we proposed a conditioning regimen consisting of the combined treatment of the small-molecule Bcl-2/Bcl-xL inhibitor ABT-737 with low-dose cyclosporine a (CsA) and one injection of a CD154-blocking antibody. This treatment was sufficient to allow engraftment of allogenic HSCs upon transplantation of a reasonable number of BM cells (20-25 mio cells), leading to low-level peripheral chimerism [191]. However, the underlying mechanisms that allowed this mixed chimerism induction remain elusive. Lymphopenia as a result of Bcl-2/Bcl-xL inhibition, and immunosuppression by low-dose CsA might have prevented rejection of allogenic cells and the development of Graft-versus-Host Disease (GVHD). Survival of HSCs has been shown to critically depend on Mcl-1, and this anti-apoptotic factor is not inhibited by ABT-737 [37, 97]. In contrast, loss-of-function studies suggest that Bcl-2 is not required for HSC maintenance [294]. Thus, Bcl-2/Bcl-xL blockade should not result in loss of HSCs. Indeed, clinical

trials have not reported myelotoxicity of navitoclax, the orally bioavailable analogue of ABT-737. Thus, we assume that this conditioning protocol based on ABT-737 and CsA treatment, does not involve elimination of endogenous HSCs, which is remarkable. Understanding the mechanisms that are involved in the engraftment process of transplanted HSCs without creating „space“ in the BM environment might help to further develop low-toxicity conditioning protocols. In the present study, we embarked on studying the effects of ABT-737 (with or without CsA) on the early hematopoietic compartment in the BM. The project is still ongoing, thus I will present the different approaches we took so far, trying to understand the phenomenon, comment on yet open questions, limitations and planned experiments.

Materials and Methods

Mice

C57BL/6 (B6, Ly5.2), B6.Cg-FoxP3^{tm1Mal} (FoxP3-GFP), B6-Tg(UBC-GFP)^{30Scha} (GFP), C57BL/6-Ly5.1 (Ly5.1) and Ly5.1xLy5.2 F1 mice were housed under pathogen-free conditions at the University of Zürich and used for experiments at age 8 to 16 weeks, always in age and sex matched groups. FoxP3-GFP mice express eGFP under the control of the FoxP3 transcription factor [267] and were kindly provided by Bernard Malissen (Centre d'Immunologie de Marseille-Luminy, France). With ageing, these mice seem to express higher percentage of Tregs. For this reason, FoxP3-GFP mice were always perfectly age-matched within one experiment. All animal experiments were performed according to protocols approved by the local legal authority (Veterinary office, Canton of Zürich, Switzerland).

Fluorescent activated cell sorting (FACS)

FACS analyses were performed on a LSR II Fortessa (Becton Dickinson). The following antibodies were used to stain 1x 10^6 cells of thymus or peripheral blood: CD4 (GK1.5) -PE, -AF700, B220 (RA3-6B2) -PerCP-eFluor710, CD11b (RA3-6B2) -PE-Cy7, FoxP3 (FJK-16s) -biotin, streptavidin-APC (all from eBioscience), and CD8 (53-6.7) -APC-Cy7, CD45.1 (A20) -APC, CD45.2 (104) -FITC (all from BioLegend). For BM stainings, the following antibodies were used: Ly6A/E (sca-1, D7) -BV421, CD16/32 (93) -APC, CD127 (IL-7Rα, A7R34) -BV605, CD135 (A2F10) -PE, CD150 (SLAM, TC15-12F12.2) -BV650 and lineage cocktail in AlexaFluor700 (all from BioLegend), and CD117 (c-kit, 2B8) -PerCP-eFluor710, CD34 (RAM34) -biotin, -eFluor450, streptavidin-PE-Cy7 (all from

eBioscience). For live-dead discrimination PI, 7-AAD or Zombie Aqua fixable viability kit (BioLegend) were used. Mice were bled sublingual and chimersim was analyzed in peripheral leukocytes at different time points after BM transplantation, and donor-derived cells were identified by endogenous expression of GFP or by staining of congenic markers. For intracellular stainings, a FoxP3 staining buffer set (eBioscience) was used according to the manufacturer's instructions.

In vivo drug treatment and BM transplantation

For in vivo application, ABT-737 was dissolved in polyethylene glycol, Tween 80, DMSO and dextrose solution and injected i.p. at 50 mg/kg. CsA (Enzo Life Sciences) was dissolved in a Cremaphor EL/ethanol solution, then diluted in HBSS and injected s.c. at 10 mg/kg. Control mice were treated with the corresponding vehicles. For short term ABT-737 treatment, mice received 5 ABT-737 injections from day -3 to day -1, before their BM was harvested and analysed on day 0. The ABT-737 mixed chimerism protocol was described in detail previously [191]. The conditioning of mice consisted of ABT-737 and CsA treatment with a total of 5 injections on days -3 to -1, and 1 daily injection until day 11, or ABT-737/CsA alone plus vehicle of the second drug, or vehicle only. On day 0, 20x 10^6 donor BM cells suspended in Media 199 containing 10 mM HEPES, 10 µg/ml DNAse and 4 µg/ml Gentamycin (called BM medium), were transplanted by tail vein injection and peripheral blood chimerism was followed over time.

Blood transplantation

To investigate whether ABT-737 and CsA treatment results in HSC mobilisation, a modified approach described in [292] was used. Donor mice (Ly5.1) received short time ABT-737 + CsA, or vehicle treatment (see above). The next day, mice were terminally bled by heart puncture, and blood of mice from the same treatment group was pooled in EDTA-coated tubes (the same volume of blood for the two treatment groups was collected). The blood was washed twice in BM medium (see above), resuspended in medium, and a low amount of EDTA was added in order to prevent clotting. Ly5.2 BM cells were added to cell suspension (in a concentration that resulted in 0.2 mio cells per recipient) and 500 µl of BM cell/blood mixture was injected i.v. into lethally irradiated recipients (900 cGy, Ly5.1xLy5.2). Based on the initial volume of blood, each recipient received 250 µl Ly5.1 blood.

Thrombopoietin measurement

Mice received short time ABT-737 +/- CsA, or vehicle treatment, and were bled 16h af-

ter the last injection. Serum thrombopoietin was measured with a Quantikine ELISA kit according to manufacturer instructions (RnD Systems).

Statistics

Differences between treatment groups were assessed by one-way Anova followed by Turkey test for multiple comparison. In animal experiments, each data point represents results from one single mouse. $P<0.05$ was considered significant, *$P<0.05$; **$P<0.01$; ***$P<0.001$, n.s., not significant. Graph Pad Prism Software Version 5.0 was used for calculations.

Results

Synergism of combined ABT-737 and CsA treatment in syngeneic BM transplantation

To solely focus on the effects of ABT-737 and CsA on HSC engraftment, while excluding any immunological processes such as rejection of transplanted cells, we chose to perform BM transplantation in syngeneic strain combinations.

A first syngeneic BM transplantation experiment aimed at assessing the importance of the individual components of the ABT-737 tolerance protocol [191]. We treated recipient animals for two weeks with ABT-737 and CsA, ABT-737 alone or CsA alone or only with vehicle, and 20 mio BM cells were injected on the fourth day after starting the treatment. A two-week treatment with vehicle did not result in syngeneic mixed chimerism (Figure 2.12). Presumably, 20 mio cells of whole BM suspension were not sufficient to efficiently compete for niches in unmanipulated hosts. Similarly, low-dose CsA alone did not lead to engraftment of transplanted BM cells. In contrast, ABT-737 treatment resulted in much higher chimerism levels ranging between 3-4% in T cells and approximately 10% in B cells and monocytes. Surprisingly, combination of the two drugs led to an even further increase of chimerism (Figure 2.12). Synergism between ABT-737 and CsA has been shown previously [243]. CsA reduced the half-maximal inhibitory concentration of ABT-737 in vitro in a concentration dependent manner and strongly reduced Bcl-2 expression in lymphocytes. This resulted in significantly decreased lymphocyte numbers in vivo when the two drugs were combined, as compared to the monotherapies [243]. Interestingly, we observed a loss of myeloid chimerism in ABT-737 treated animals at more than one year after transplantation (the same was observed in a repetition experiment, Figure 2.12 D). We plan to keep these mixed chimeras for further long-term analysis. If in ABT-737 treated animals chimerism is

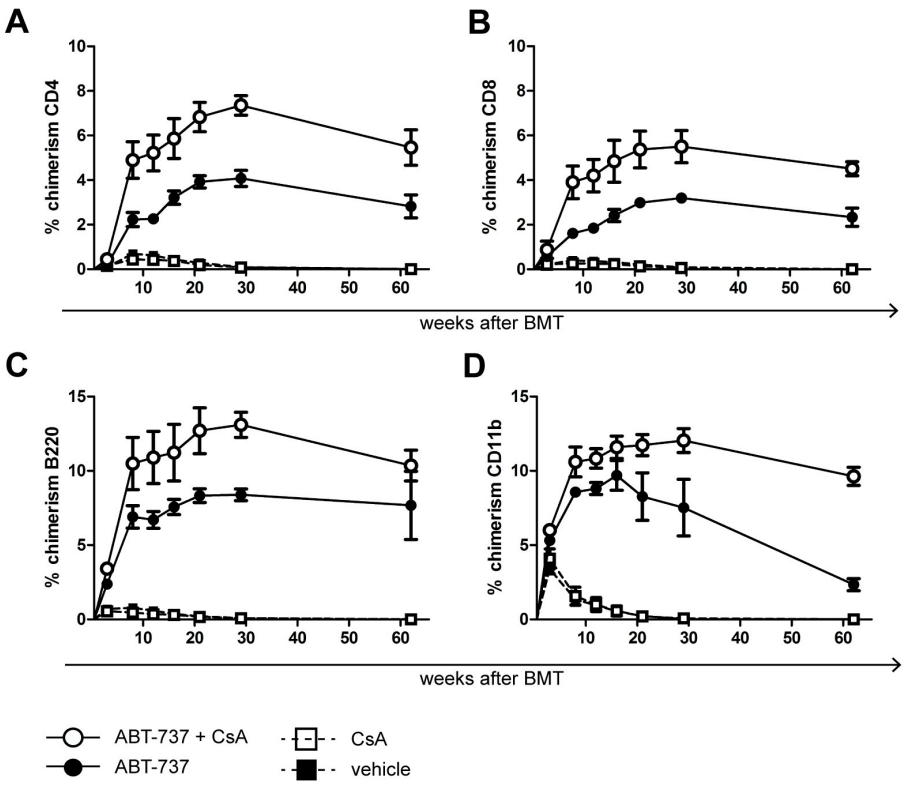

Figure 2.12: ABT-737 treatment promotes syngeneic chimerism
(A-D) Mice were treated for 2 weeks with either ABT-737 and CsA (white circle), ABT-737 alone (black circle), CsA alone (white square) or vehicle (black square) and transplanted with syngeneic BM. Peripheral blood chimerism for **(A)** CD4, **(B)** CD8, **(C)** B220 and **(D)** CD11b was measured at indicated time points, n = 7 mice/group and data represent mean +/- s.e.m.

also lost in other lineages, this might indicate that true HSCs failed to engraft upon transplantation under ABT-737 monotherapy, and that rather very early progenitors sustained chimerism until their self-renewal potential was exhausted. Analysis of the thymuses of such long-term chimeras more than one year after transplantation indeed pointed towards loss of T cell chimerism in ABT-737-only treated animals, however DN thymocytes of ABT-737 and CsA treated animals have also lost chimerism (Figure 2.13).

To closer address the question whether we have engraftment of true HSCs, we performed a serial transplantation experiment. Mice were transplanted with syngeneic BM under treatment with either ABT-737 plus CsA, ABT-737 alone, CsA alone or vehicle, and chimerism was followed over 19 weeks (Figure 2.13 F-I; bars show chimerism level

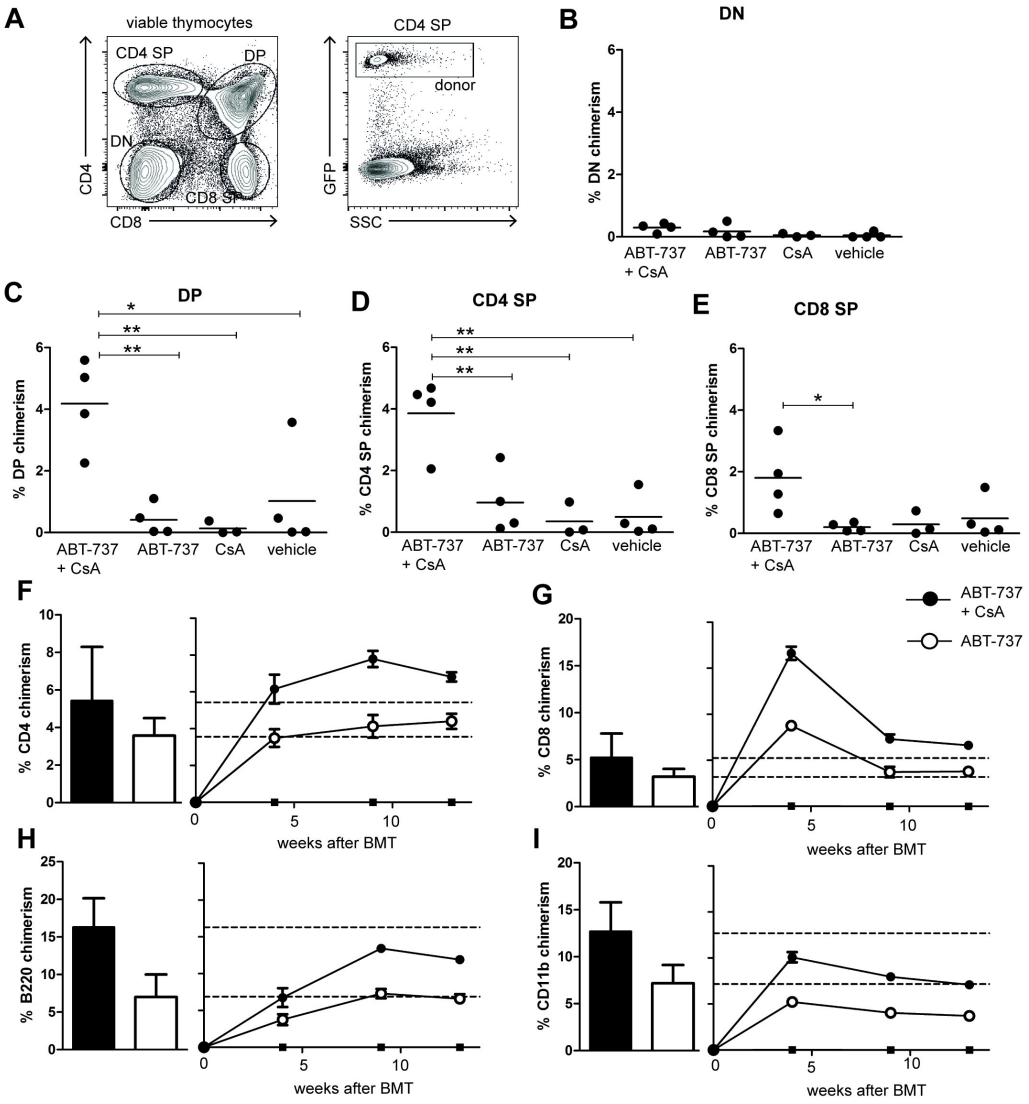

Figure 2.13: Assessment of long-term repopulation potential of transplanted cells
(A-I) Mice were treated for 2 weeks with either ABT-737 and CsA, ABT-737 alone, CsA alone or vehicle, and transplanted with syngeneic BM. More than one year after transplantation, thymuses where harvested. **(A)** Representative FACS plot showing gating for thymocyte populations and chimerism based on GFP expression. Dot plots showing chimerism levels of **(B)** DN, **(C)** DP, **(D)** CD4 SP and **(E)** CD8 SP cell populations, n = 3-4 mice/group. **(F-I)** In a similar experiment, 19 weeks after transplantation, BM was harvested and transplanted into lethally irradiated recipients. **(F)** CD4, **(G)** CD8, **(H)** B220 and **(I)** CD11b chimerism; bars show chimerism of donor mice at time of harvest and curves show chimerism after transplantation into secondary recipients. ABT-737 and CsA treated animals (black bars/circles) and ABT-737 only treated animals (white bars/circles), n = 3 mice/group for initial treatment and n = 6 mice/group secondary transplantation and n = 2 mice/group for recipients of BM from CsA and vehicle treated animals without chimerism. Data show mean +/- s.e.m. and differences were assessed with one-way Anova followed by Turkey test.

at harvest). BM of these mixed chimeras was mixed and transplanted into lethally irradiated recipients, and chimerism was followed again over time. Vehicle and CsA groups did not have any chimerism at time of transplantation, but their BM was transplanted as negative control (Figure 2.13 F-I; black squares). Interestingly, CD11b chimerism was in both chimeric groups lower than expected, while T cell chimerism was slightly higher. These mixed chimeras are still available and upon measurement of long-term chimerism, another round of transplantation into irradiated recipients will be performed. Hopefully, this will clarify the question of whether the transplanted cells possess long-term repopulation potential and thus can be considered as true HSCs.

One point is worth noting when looking at the above described syngeneic transplantation experiments: as donor strain we have always used B6.UBC-GFP mice that the contain freely floating GFP in the cytosol of all their cells. Theoretically, there might be low-grade rejection of these transplanted cells, and the positive effects of ABT-737 and CsA might be attributed to their immunosuppressive action. However, other experiments that we further performed, suggest synergistic effects of ABT-737 and CsA beyond their immunosuppressive effects.

ABT-737 and CsA treatment does not recruit HSCs into peripheral circulation

Based on the observation that Bcl-2/Bcl-xL inhibition promotes engraftment of transplanted HSCs, we reasoned that such treatment might disrupt the HSC niche and promote mobilisation of endogenous HSCs into peripheral blood. Transplanted and endogenous HSCs could then compete simultaneously for the increased number of open niches, resulting in higher chimerism compared to situations where no mobilization takes place. To test this hypothesis, we designed an experiment where Ly5.1 mice were treated with either ABT-737 and CsA or vehicle for three days. Their blood was isolated and transplanted along with a low number of competitor BM cells (Ly5.2) into lethally irradiated F1 (Ly5.1xLy5.2) recipients (Figure 2.14 A). A similar approach was used previously to prove that few HSCs can be found in the periphery [292]. If treatment indeed would result in HSC mobilization, increased chimerism levels could be expected upon transplantation of the blood. Analysis of peripheral blood of recipients however disproved this hypothesis, as chimerism was lower in treated animals (Figure 2.14 B-E). For lymphocytes, at least the initially lower levels of chimerism are not surprising, as many peripheral lymphocytes are present

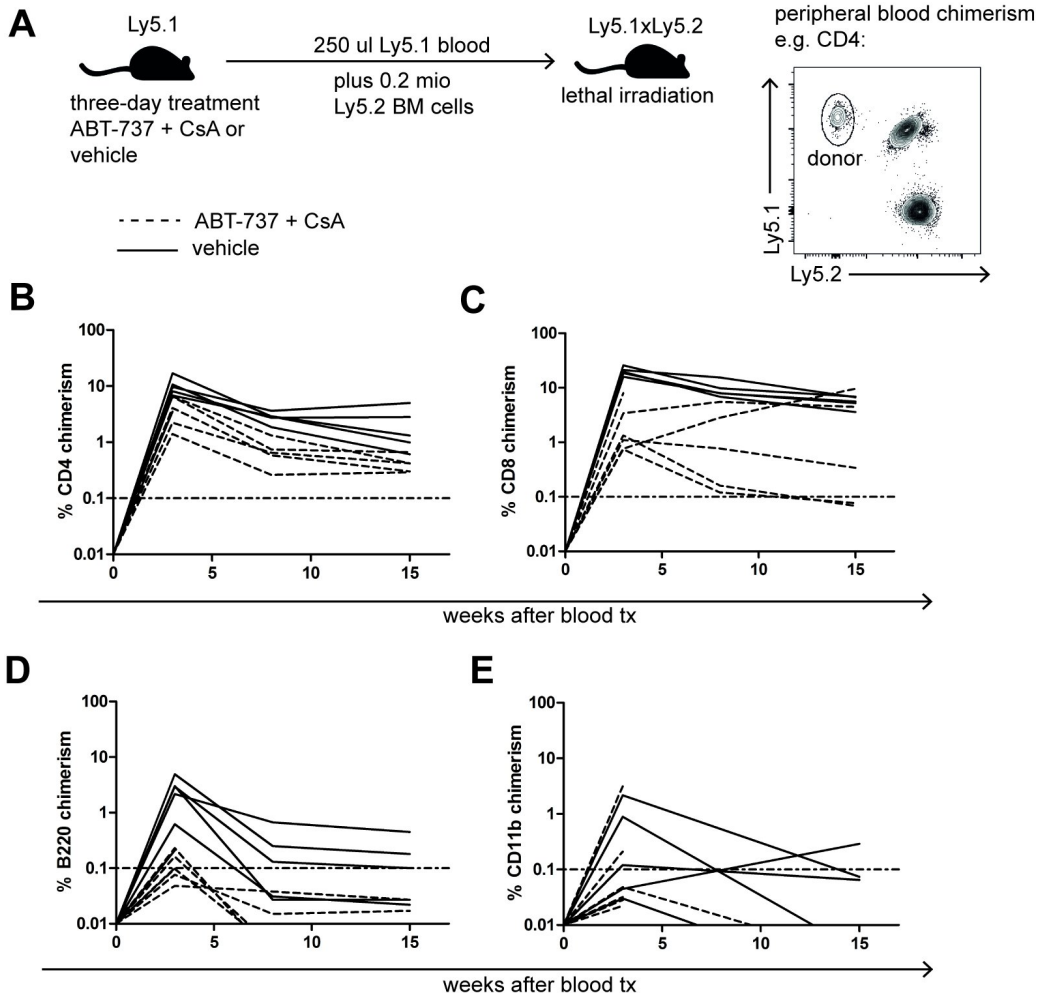

Figure 2.14: ABT-737 and CsA treatment does not mobilize HSCs into peripheral circulation **(A)** Experimental setup: Ly5.1 donors were treated for three days with either ABT-737 + CsA or vehicle (total 5 injections), the next day their blood was isolated, lysed, washed and transplanted along with 0.2 mio Ly5.1 competitor BM into lethally irradiated F1 (Ly5.1xLy5.2) recipients. FACS plot shows gating strategy for chimerism detection (gated on CD4+ cells), double positive cells are irradiation-resistant recipient-derived T cells. **(B)** CD4, **(C)** CD8, **(D)** B220 and **(E)** CD11b chimerism. Horizontal dashed line represents detection limit and lines represent individual mice (n = 5-6/group).

in the transplanted blood, and ABT-737 + CsA greatly depletes these cells. Again, these mice are still available and a long-term chimerism measurement will be performed to definitely confirm lower chimerism in mice receiving blood from ABT-737 + CsA treated animals. In contrast to the syngeneic transplantation experiments where GFP-expressing donor cells were used, in this experimental setting rejection of transplanted cells can be excluded.

Bcl-2/Bcl-xL inhibition in combination with CsA increases HSCs by absolute and relative means

The above negative findings prompted us to directly look at the BM, in order to detect effects of the treatment in the stem cell and progenitor compartment. Again, mice were subjected to the four different treatment regimen for three days before their BM was analyzed by FACS the following day. Figure 2.15 shows the gating strategy used for the detection of progenitor populations and HSCs.

After gating for singlets and viable cells, all lineage positive cells (T and B lymphocytes, NK cells, monocytes/macrophages, granulocytes and erythrocytes) were excluded and only lineage negative/low cells were used for further analysis. Among the c-kit+ sca-1- population, different progenitors can be identified based on their CD16/32 and CD34 expression (CMP common myeloid progenitors, giving rise to GMP granulocyte-macrophage progenitors and MEP myeloid-erythroid progenitors). Unfortunately, the staining for common lymphoid progenitors (CLPs) repetitively failed. Among the c-kit+sca-1+ population (called LSKs), fractions enriched for LT-HSCs and ST-HSCs/MPPs, respectively, can be discriminated based on their Flt-3 expression [295]. Furthermore, SLAM-HSCs are a subgroup of LT-HSCs that additionally express CD150 and are considered to display an even higher self-renewal potential than LT-HSCs [287].

The combined treatment of ABT-737 and CsA resulted in a slightly reduced total cell count per tibia, as mature lymphocytes were probably killed in the BM, similarly to the periphery (Figure 2.16 A). This reduction in lineage positive cells was sufficient to result in a significant relative enrichment of lineage negative cells in the BM (Figure 2.16 B). Among these lineage negative cells, a further enrichment of LSKs was observed (Figure 2.16 C). Taken together, ABT-737 plus CsA treatment resulted in a slight shift of BM cells towards less differentiated populations.

Intriguingly, SLAM-HSCs highly significantly increased by absolute and relative

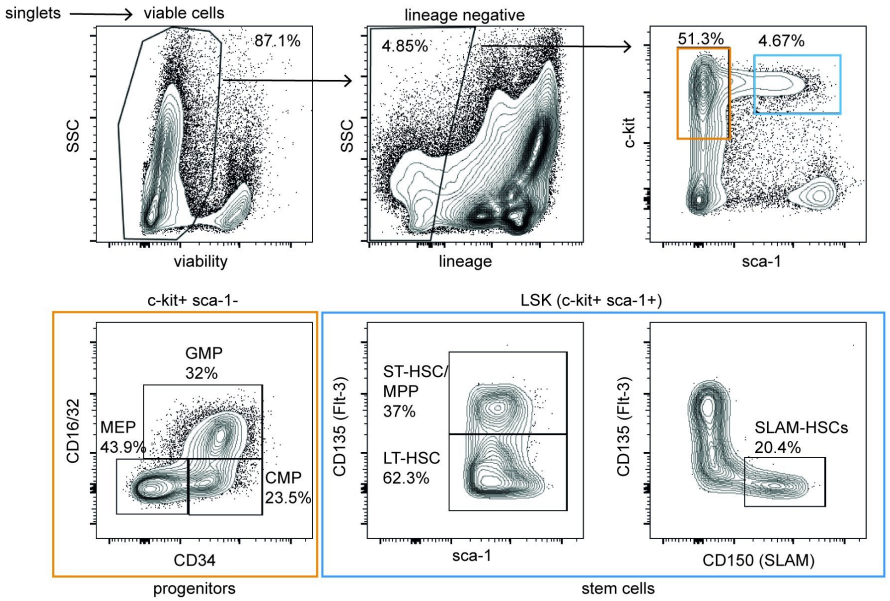

Figure 2.15: Gating strategy for HSCs and progenitor populations
After gating on singlets, viable cells are selected. A cocktail consisting of anti-CD3, -Gr-1, -CD11b, -B220 and -Ter-199 antibodies allows to gate on lineage negative/low cells within the BM. Myeloid progenitor populations can be discriminated based on their CD16/32 (IgG Fcγ receptor II/III) and CD34 expression. c-kit+ sca-1+ LSKs can be divided into LT- and ST-HSCs/MPPs based on their CD135 (Flt-3) expression. SLAM-HSCs are even higher in the hematopoiesis hierarchy and are discriminated from LT-HSCs by their additional CD150 (SLAM) expression. Gating according to [295].

means upon a three-day combined drug treatment (Figure 2.17 A, B). This indicates that HSCs with a high self-renewal potential not only were resistant towards Bcl-2/Bcl-xL inhibition, as suggested based on the literature, but that they expanded upon treatment. The same effect could be seen in LT-HSCs, but less pronounced, while ST-HSCs/MPPs changed neither by absolute nor relative means (Figure 2.17 C, D).

Finally, we compared differences of progenitor populations upon drug treatment. While CMPs were not affected by ABT-737 and/or CsA treatment, a significant shift from MEPs towards GMPs was observed (Figure 2.18). Loss of MEPs was not unexpected, as megakaryocytes are known to depend on Bcl-xL for their survival [296, 297]. The shift towards the granulocyte/monocyte lineage might provide an explanation for the high CD11b chimerism observed in both, syngeneic and allogeneic transplantation upon ABT-737 treatment [191]. Figure 2.18 D provides an overview on how

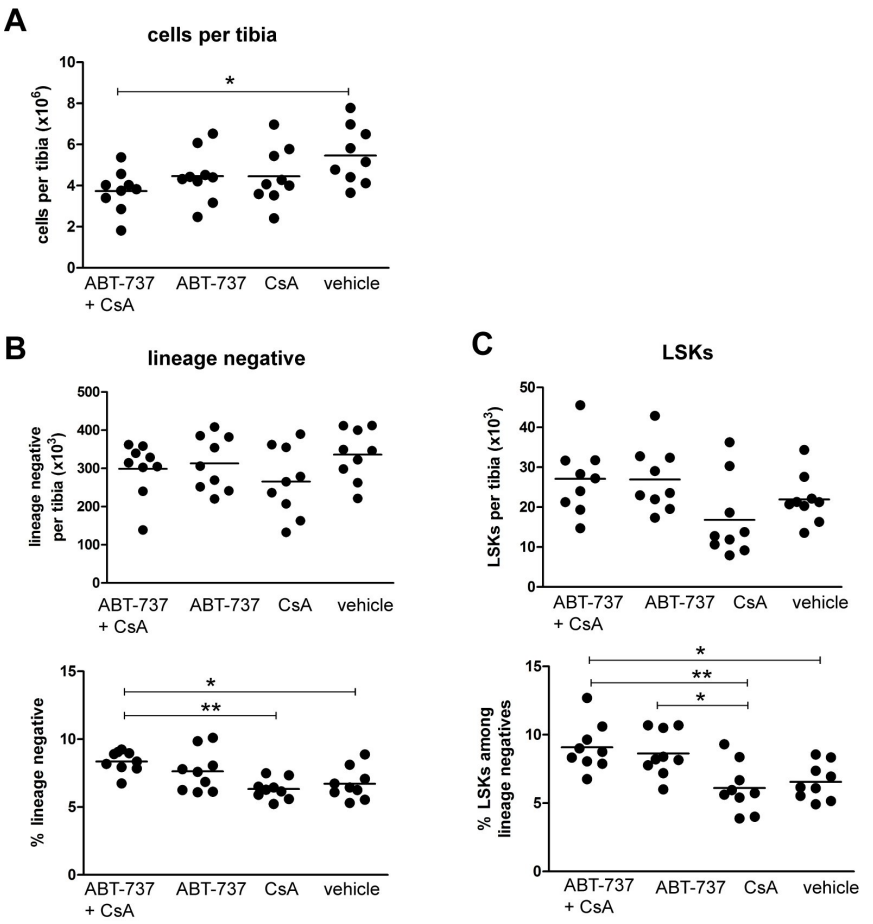

Figure 2.16: Enrichment of LSKs upon ABT-737 and CsA treatment
(A-C) Mice were treated for three days with either ABT-737 and CsA, ABT-737 alone, CsA alone or vehicle (total 5 injections) and BM was analyzed by FACS the next day. Dot plots show the **(A)** total cells per tibia, the absolute and relative count of **(B)** lineage negative population and **(C)** the LSK population. Pooled data of two independent experiments, n = 9 mice/group. Differences between treatment groups were assessed with one-way Anova followed by Turkey test.

HSCs and progenitors are affected by ABT-737 + CsA treatment. To further investigate the effect of ABT-737 on progenitor populations, we plan to perform in vitro colony forming assays, which will allow us to test directly resistance of GMPs to Bcl-2/Bcl-xL inhibition. Furthermore, colony forming assays with BM cells from transplanted mice will allow us to compare the abundance of donor-derived progenitors in the BM by monitoring GFP+ colonies.

Does treatment indirectly affect HSC expansion?

As explained in the introduction, TPO is a cytokine/hormone that is important for HSC homeostasis and maintenance by directly interacting with its receptor c-mpl expressed on HSCs. Bcl-xL inhibition results in thrombocytopenia, thus ABT-737 treatment is expected to result in increased TPO levels, that in turn might promote the observed HSC expansion [43]. To test this hypothesis, we

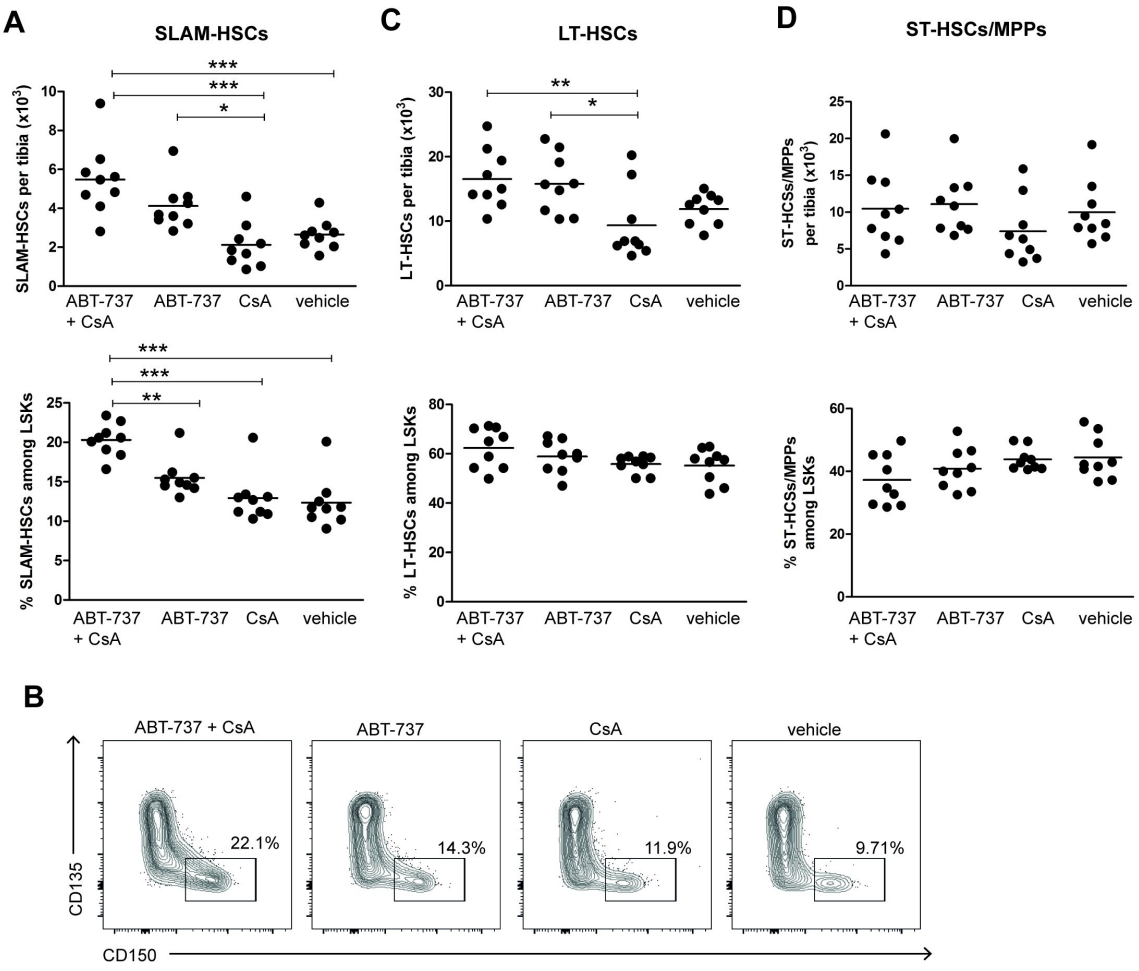

Figure 2.17: Relative and absolute enrichment of HSCs upon Bcl-2/Bcl-xL inhibition
(A-D) Mice were treated for three days with either ABT-737 and CsA, ABT-737 alone, CsA alone or vehicle (total 5 injections) and BM was analyzed by FACS the next day. Dot plots show the absolute and relative cell counts of **(A)** SLAM-HSCs **(C)** LT-HSCs and **(D)** ST-HSCs. **(B)** shows representative FACS plots illustrating the enrichment of SLAM-HSCs among LSKs in the different treatment groups. Pooled data of two independent experiments, n = 9 mice/group. Differences between treatment groups were assessed with one-way Anova followed by Turkey test.

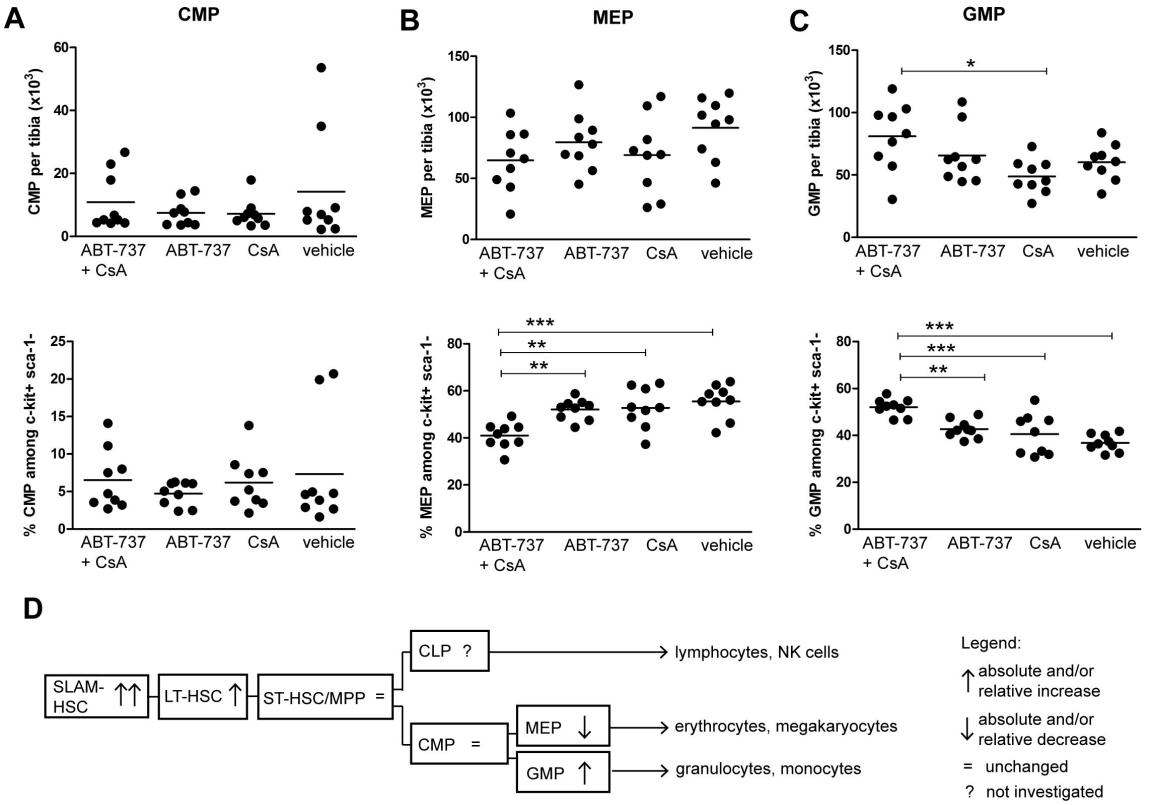

Figure 2.18: MEP are sensitive towards Bcl-2/Bcl-xL inhibition
(A-C) Mice were treated for three days with either ABT-737 and CsA, ABT-737 alone, CsA alone or vehicle (total 5 injections) and BM was analyzed by FACS the next day. Dot plots show the absolute and relative cell counts of **(A)** CMP, **(B)** MEP and **(C)** GMP. **(D)** schematic summary of the findings regarding the various stem cell and progenitor populations. CMP common myeloid progenitor, MEP myeloid-erythroid progenitor, GMP granulocyte-monocyte progenitor. Pooled data of two independent experiments, n = 9 mice/group. Differences between treatment groups were assessed with one-way Anova followed by Turkey test.

treated mice for three days with ABT-737 +/- CsA, and compared the TPO concentration in the sera of these mice with vehicle treated animals. To our surprise, TPO was significantly reduced upon combination treatment (Figure 2.19 A). This finding will certainly require further investigation.

Our previous observation, that Tregs increase by relative means upon treatment, was confirmed in the BM (Figure 2.19 B,C). In fact, it is known that the percentage of Tregs among CD4 cells is much higher in the BM than in the periphery, and it has been suggested that the BM is a reservoir for Tregs [298, 299]. However, not much is known about the function of these Tregs in the BM. It will be interesting to assess, whether these Tregs are involved in the engraftment process of transplanted HSCs.

Discussion

The present study is based around the finding that short-time treatment with a pro-apoptotic drug in combination with an immunosuppressant allows the induction of mixed hematopoietic chimerism without myelosuppression in both, the allogeneic and syngeneic setting [191]. Understanding the mechanisms that allow engraftment of transplanted HSCs into such minimally conditioned hosts is important, especially also as the concept of apoptosis modulation in this context is innovative and completely unexplored to date. ABT-737 reduces precursor frequency of allo-reactive naïve and memory T cells, restores sensitivity to costimulation blockade in tolerance induction protocols [300] and at the same time spares Tregs, thereby shifting the balance between effector T cells and Tregs towards tolerance (Section 2.2). All these positive effects may suggest ABT-737 (or its orally available analogue navitoclax) to be a valuable novel drug in the transplantation setting. Assuming a conditioning regimen of transplant reci-pients that undergo combined BM and kidney transplantation; navitoclax may be useful as short-time treatment for preparing the immune system for the transplantation, e.g. replacing ATG, and at the same time it may render the body (or the BM microenvironment) in a status that favours the engraftment of transplanted cells. Navitoclax has indeed been tested in phase I and II clinical cancer trials as single drug and in combination therapies, and the drug has generally been well tolerated by patients, with the only side effect of thrombocytopenia [283].

This project is still ongoing and various hypotheses, that may explain the positive effects of ABT-737 and CsA treatment for HSC engraftment, have been established and

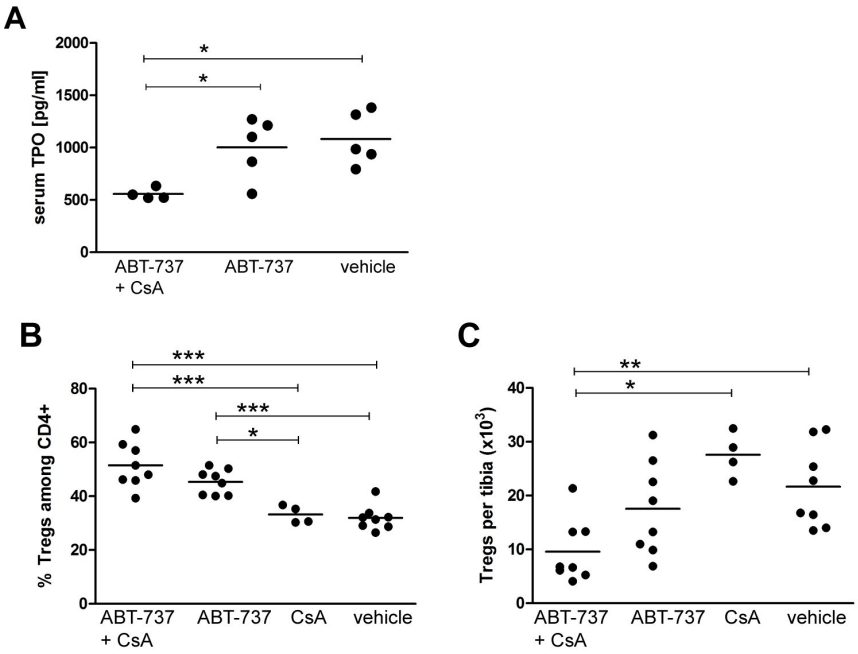

Figure 2.19: Assessment of TPO in serum and Tregs in BM
After three days of drug treatment, serum and BM were analysed. **(A)** serum TPO concentration 16h after the last injection, **(B)** relative and **(C)** absolute Treg counts in the BM. n = 4-5 mice/group for TPO measurement and n = 8 mice/group for Treg measurement, pooled data of two independent experiments. Differences between treatment groups were assessed with one-way Anova followed by Turkey test.

tested. Thereby, we came across some fascinating and unexpected findings, for example the expansion of SLAM-HSCs or the significant decrease of TPO upon treatment. It is difficult to draw a well-defined red line along the results at this stage of the project, thus the manuscript rather gives an overview about all the approaches undertaken so far, including the ones that failed or are puzzling.

Various experiments are planned and/or in preparation to closer investigate certain findings. Very important will be to test whether treatment results in proliferation of HSCs, since this would be expected for populations that increase in absolute numbers. If these cells proliferate indeed, what drives them to do so? I am convinced that blockade of anti-apoptotic factors does not directly influence the behaviour of HSCs, but that rather indirect mechanisms come into play. In fact, I was absolutely convinced that the profound thrombocytopenia induced by the combination treatment would result in high TPO levels, which then in turn would act on HSCs [243]. It was assumed that TPO promotes quiescence of HSCs rather than proliferation [301, 302]. However, recent research questions this dogma. I recently learned about data presented at the Zürich Immunology Meeting, that TPO mimetics recruit quies-

cent HSCs into proliferation (unpublished). During steady state, basal TPO signalling probably promotes quiescence, while it expands HSCs under stress conditions [303]. The significant decrease of TPO upon ABT-737 and CsA treatment was unexpected and for this reason, TPO levels in the serum need to be followed at regular intervals, as there might be an oscillation of TPO levels. To closer evaluate the potential role of TPO in promoting HSC engraftment, transplantation experiments under TPO mimetic treatment might be performed. It has been shown that post-transplantation HSC expansion highly depends on TPO [301, 304].

Another aspect that we would like to address is the involvement of Tregs in the engraftment process. In the allogeneic setting, it has been shown in several studies that co-infusion of Tregs together with BM cells promoted chimerism induction under low-toxicity conditions [190, 305]. In these studies however, focus was given rather at tolerance mechanisms once chimerism was established, than at the engraftment process per se. An important study published in Science has provided evidence that Tregs closely associate with stem cells in the BM, providing an immune-privileged site for transplanted allo-HSCs in unconditioned hosts [306]. Furthermore, a recent study has shown that recipient Tregs are required for long-term HSC engraftment, and that they promoted proliferation of HSCs in a reduced-toxicity protocol [307]. As we previously found that Tregs are needed for successful transplantation of allo-HSCs in the ABT-737 tolerance protocol (Section 2.2), it would be very interesting to test whether the strong relative increase of Tregs in the BM upon ABT-737 treatment is functionally relevant also in syngeneic transplantation. Thus, we would like to test whether Tregs are needed beyond their immunosuppressive action, and whether they are involved in HSC expansion upon treatment. For such experiments, we can take advantage of the DEREG mice that we are breeding and that allow specific depletion of Tregs.

Contribution of S.S.G. to the manuscript

Designed and performed all experiments independently, prepared figures and wrote the manuscript.

2.4 Bone marrow stem cell transplantation alleviates Fanconi syndrome caused by a membrane bound transporter

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Status of the manuscript: project in finalization

Abstract

Dent's disease is caused by a genetic defect in a chloride-proton exchanger located in early endosomes of proximal tubular cells of the kidneys. This defect results in dysfunctional receptor-mediated endocytosis, and as a consequence in severe manifestations of Fanconi syndrome. In the present study, we evaluated the potential of hematopoietic stem cell transplantation in this disease. Clcn5 KO mice, the homologue animal model for Dent's disease, were transplanted with healthy bone marrow cells. This treatment resulted in significant improvement of renal Fanconi syndrome four months after transplantation. Engrafted hematopoietic lineage-derived cells closely associated with proximal tubuli and importantly, megalin scavenger receptors were re-expressed at the apical brush border membrane of proximal tubular cells. Furthermore, Clcn5 gene expression in transplanted kidneys significantly correlated with improvement of Fanconi syndrome. Our study thus provides evidence that bone marrow transplantation may partially rescue the endocytic dysfunction caused by genetic defects of a membrane bound transporter.

Introduction

Stem cell therapy has great therapeutic potential in the regenerative and therapeutic setting and is attracting much attention for a wide range of conditions. Bone marrow (BM) derived stem cells comprising both, hematopoietic stem cells (HSCs) and mesenchymal stromal cells (MSC), displayed reparative and angiogenic properties in experimental models of acute kidney injury, such as ischemia/reperfusion injury, cisplatin-induced acute kidney injury or anti-Thy1.1 glomerulonephritis [173, 206–208]. In such models, the local or systemic infusion of MSCs exerted protective effects by either paracrine or endocrine mechanisms [206, 207]. However from a clinical perspective, the treatment of progressive chronic kidney diseases might be of greater importance than improvements in these somewhat „artificial“ injury models. Interesting in that regard, BM transplantation has been shown to be successful in reducing disease burden in a pre-clinical model of cystinosis [212, 308]. This disease is caused by defects of the CTNS gene that encodes a membrane bound transporter, resulting in multi-organ dysfunction due to accumulation of cysteine crystals. Kidneys are severely involved in this disease, and patients develop progressive kidney failure already in childhood [309]. The transplantation of healthy BM cells improved several serum and urinary parameters in diseased mice, and although the mechanism of how healthy cells derived from the hematopoietic lineage can improve such a disease remains somewhat obscure, the findings of these cystinosis studies may pave the path to treatment of many other diseases underlying defective membrane-bound enzymes or transporters.

Dent's disease is a hereditary kidney disease caused by inactivating mutations of the ClC-5 2Cl-/H+ exchanger. The disease is X-linked and progresses to end-stage renal disease in a majority of affected males [229]. ClC-5 is highly expressed at the membrane of early endosomes in proximal tubule (PT) cells, the thick ascending limb and in α-intercalated cells of the collecting duct [222]. Mouse models with Clcn5 knockout genes recapitulate well the human disease phenotype and allowed detailed studies of the pathomechanism in Dent's disease [226, 310]. ClC-5 has been shown to be necessary for acidification of early endosomes and Cl- conductance, and lack of either results in low-molecular weight (LMW) proteinuria, glycosuria and hypercalciuria [225, 226, 310, 311]. These manifestations of Fanconi syndrome are caused by loss of megalin and cubilin at the apical membrane of PT cells [224].

Based on the encouraging results of BM transplantation in cystinosis, we aimed at providing a proof-of-concept that this treatment is effective also in another inherited nephropathy with a different pathogenesis, such as Dent's disease.

Materials and Methods

Mice

ClC-5 KO males ($Clcn5^{-/Y}$) and WT littermate controls ($Clcn5^{+/Y}$) were used for experiments. These mice were generated by deletion of exon VI and kindly provided W. Guggino (John Hopkins) [226]. BM donors expressed enhanced GFP under the direction of the human ubiquitin C promotor (UBC-GFP), leading to ubiquitous GFP expression in all cells (The Jackson Laboratory). All mice were on C57BL/6 background and only males were used. Mice were bred and housed under specific pathogen-free conditions at University of Zurich and all animal experiments were performed according to protocols approved by the local legal authority (Veterinary office, Canton of Zürich, Switzerland).

Bone marrow transplantation

ClC-5 KO and WT littermate controls were lethally irradiated (900 cGy) with a Cs source and reconstituted approx. 6 hours later by tail vein injection of 10x 10^6 unfractionated BM cells suspended in Media 199 containing 10 mM HEPES, 10 µg/ml DNAse and 4 µg/ml Gentamycin. BM cells were either isolated from UBC-GFP or ClC-5 KO donors aged 8-14 weeks. Recipients were randomly distributed among treatment groups and transplanted at the age of 10 weeks. Peripheral blood chimerism was measured at time of harvest. Cells were stained for CD4, CD8, CD11b and B220 and acquired at FACS Canto II or LSR II Fortessa.

Urinary parameters

Urine samples were collected overnight (12h) in metabolic cages with ad libitum access to food and drinking water one week prior BM transplantation and 16 weeks after transplantation. Urinary calcium, glucose, and creatinine were measured with a Synchron CX3 Delta System (Beckman Coulter) and urinary CC16 was measured by latex immunoassay [312].

Collection of kidneys

Mice were anesthetized using an isoflurane vaporizer and perfused through the right ventricle with a 0.9% NaCl solution containing heparin (5000 U/ml), 1% Procain-HCL (Lidocain) and 16% $CaCl_2$. Then, the left kidney was clamped and the tissue fixed by infusion of 3% PFA dissolved in 0.1M Na-cacodylate. One half of the unfixed kidney was used for protein isolation and the other

half for RT-qPCR analysis. The fixed kidney was kept for 1h in 3% PFA on ice, then in 1% PFA overnight. After washing in PBS and soaking in 30% sucrose, the kidneys were embedded in OCT medium.

Quantitative real-time PCR

Total RNA was extracted from half kidney using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio Rad) according to manufacturer's protocol. DNAse I treatment was performed to eliminate genomic DNA contamination. 1 µg RNA was used to perform the reverse transcriptase reaction with iScript cDNA Synthesis Kit (Bio Rad).

Changes in target gene mRNA levels were determined by relative RT-qPCR with a CFX96 Real-Time PCR Detection System (Bio-Rad), using iQ SYBR Green Supermix (Bio-Rad) for detection of single PCR product accumulation. RT-qPCR analyses were performed in duplicate with 100 nM of sense and anti-sense primers in a final volume of 20 µl. Specific primers were designed using Primer3 [313]. Primers were as followed: ClC-5 sense primer 5'-TGG AGG AGC CAA TCC CTG GTG T-3' and ClC-5 anti-sense primer 5'-AGA AAG CAT CGC TCA CAC TG-3', GFP sense primer 5'-CCA CAT GAA GCA GCA GGA CTT-3' and GFP anti-sense primer 5'-GGT GCG CTC CTG GAG GTA-3', and reference gene Gapdh sense primer 5'-TGC ACC ACC AAC TGC TTA GC-3' and Gapdh anti-sense primer 5'-GGA TGC AGG GAT GGG GGA GA-3'. PCR conditions were 95°C for 3 min followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C.The PCR products were sequenced with the BigDye terminator kit (Perkin Elmer Applied Biosystems). The multiScreen SEQ384 Filter Plate (Millipore) and Sephadex G-50 DNA Grade Fine (Amersham Biosciences) dye terminator removal were used to purify sequences reactions before analysis on an ABI3100 capillary sequencer (Perkin Elmer Applied Biosystems). The efficiency of each set of primers was determined by dilution curves.

Antibodies

The following antibodies were used for immunofluorescence stainings and/or immunoblotting: rabbit anti-GFP (Life Technologies), rabbit anti-AQP1 (Millipore), sheep anti-megalin (a gift from Dr. P. Verroust and Dr. R. Kozyraki, Paris), rat anti-F4/80 (Life Technologies), hamster anti-CD11c (Bio-Rad), rat anti-α-SMA (Bio-Rad), mouse anti-β-actin (Sigma), rabbit anti-rab5 (Cell Signaling Technology). Secondary antibodies were labelled with Alexa488, Alexa633 or Dylight649.

Immunofluorescence microscopy

6 µm sections were blocked for 30 min with

3% BSA in PBS at room temperature, before slides were incubated with primary antibodies (1:200) overnight at 4°C. After washing, slides were incubated with secondary antibodies for 1 h at room temperature and counterstained with Dapi before mounting with ProLong Gold antifade reagent (Life Technologies). Slides were visualized with a CLSM SP8 upright Leica confocal laser scanning microscope.

Immunoblotting

Proteins were extracted from kidney that were snap-frozen and stored at -80°C after harvest. Kidneys were lysed in RIPA buffer (Life Technologies) containing protease inhibitors (Roche), followed by a brief sonication and centrifugation at 16'000xg for 1 min at 4°C. Protein concentration was determined using the BCA protein assay (Pierce). Samples were normalized and diluted in Laemmli sample buffer (Bio-Rad), and proteins were separated by SDS-PAGE and subsequently blotted onto PVDF membranes. Membranes were blocked for 30 min in 5% non-fat dry milk solution at room temperature, followed by overnight incubation of primary antibodies at 4°C. Blots were washed and incubated with peroxidase-conjugated secondary antibodies, washed again and visualized by Immun-Star enhanced chemiluminescence (Bio-Rad). Immunoblots were quantified by densitometry using ImageJ software.

Statistics

To test the effect of treatment, urinary parameters at baseline and week 16 were compared by paired Wilcoxon signed rank test. Differences between unpaired groups were calculated with Mann-Whitney test, and correlation between Clcn5 mRNA expression and urinary loss of CC16 were assessed with GraphPad Prism. $P<0.05$ was considered significant, $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, n.s., not significant.

Results

Increased engraftment of GFP+ cells into KO kidneys

To test whether BM transplantation improves the phenotypic characteristics of Dent's disease in mice, we transplanted ClC-5 KO mice with WT GFP-expressing BM cells („treated"). As controls, KO mice were transplanted with KO cells („negative controls") and WT littermates with WT GFP-expressing cells („positive controls"). Urine was collected at baseline and four months after transplantation, and urinary volume, glucose, creatinine, Ca^{2+} and Clara Cell protein (CC16) were measured (Figure 2.20 A). With the exemption of a few irradiation resistant T cells, all mice displayed full hematopoietic

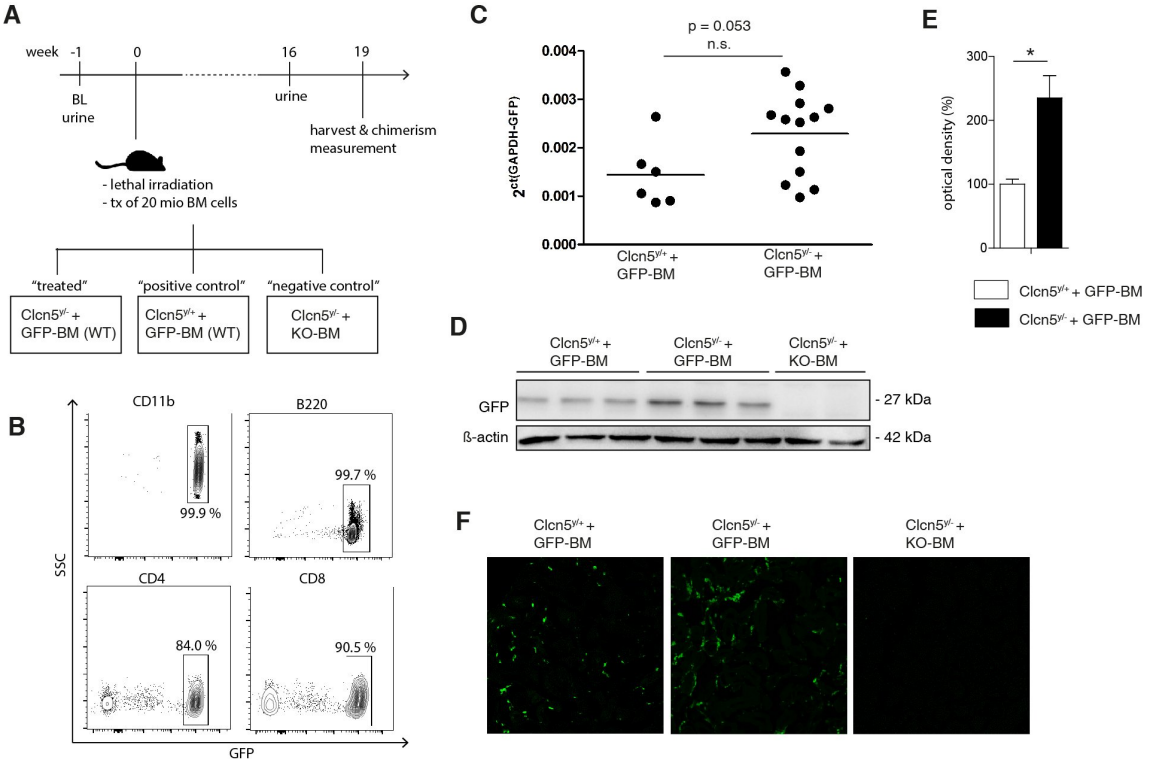

Figure 2.20: Study design and engraftment efficiency of transplanted cells
(A) Experimental setup of the study: Clcn5$^{y/-}$ (ClC-5 KO) and Clcn5$^{y/+}$ (WT) littermate controls were used as recipients. These mice were transplanted at age 10 weeks either with WT GFP+ or ClC-5 KO BM cells. Baseline (BL) urine collection (12h overnight) was performed the week before BM transplantation and again 16 weeks after BM transplantation. Peripheral blood chimerism was measured at time of kidney harvest. **(B)** Peripheral blood chimerism of monocytes, B cells and T cells, **(C)** GFP mRNA expression in WT and KO kidney of mice that received GFP+ BM cells. **(D)** western blot for GFP protein expression in kidney lysates, **(E)** quantification of optical density in GFP western blot, **(F)** confocal image showing GFP expression in kidneys. Statistical differences were assessed with Mann-Whitney test.

chimerism in peripheral blood (Figure 2.20 B). Interestingly, ClC-5 KO mice displayed higher recruitment of GFP+ cells into the kidneys than WT mice, as measured on mRNA and protein level (Figure 2.20 C-F). It has been suggested, that chronic injury is a pre-requisite to stimulate the long-term engraftment of BM-derived cells to the kidney [308].

Improvement of Fanconi syndrome in transplanted animals

Regardless of their treatment regimen, ClC-5 KO mice displayed higher values of all urinary parameters measured at baseline and at week 16 than their WT littermate controls that were transplanted with GFP BM (note; WT parameters did not change over time, Table 2.21). CC16 is a highly sensitive marker for proximal tubular dysfunction and cannot be detected in urine of healthy individuals [314]. Paired analysis showed a significant decrease of CC16 from 69.8 to 47.3 mg/g crea in treated animals. In contrast, negative controls had a significant increase in urinary CC16, pointing towards further progression of the disease over time (Figure 2.22 A). This finding was underlined by comparing the relative change of CC16 in treated and control groups (Figure 2.22 B). Urine volume did not change in treated mice, while mice transplanted with KO cells increased urinary volume further, again pointing to a progression of the disease (Figure 2.22 C). Similarly, glycosuria and Ca^{2+}/crea ratio also decreased significantly in treated animals, while it rested unchanged in negative controls (Figure 2.22 D-F). Taken together, a clear improvement of the Fanconi syndrome could be observed in mice transplanted with WT BM cells compared to negative controls that were transplanted with ClC-5 KO cells. Notably, all mice regardless of genotype and treatment regimen, had normal kidney function as measured by plasma urea and creatinine at different time points (data not shown). It has been suggested that the level of peripheral blood chimerism correlates with kidney function in cystinosis [315]. In our protocol, recipients where lethally irradiated and thus BM transplantation resulted in full hematopoietic chimerism in all mice (Figure 2.20 A). However, the engraftment level of GFP+ cells in the kidney might correlate with urinary parameters. But we did not observe a significant correlation between any of the urinary parameters and the engraftment level (data not shown).

Partial recovery of megalin expression in transplanted mice

CC16 is absorbed by receptor-mediated endocytosis in PT cells, thus the reduced urinary loss of CC16 in transplanted mice sug-

	ClC-5 KO with WT cells (treated) n = 13-15		ClC-5 KO with ClC-5 KO cells (controls) n = 8-10		WT n = 7
Urinary parameter	Baseline	Week 16	Baseline	Week 16	Week 16
Urine volume (µl/12h)	2044 ± 206.7	3907 ± 1044	2150 ± 20	6140 ± 1675	955.7 ± 159.6
CC16 (mg/g crea)	69.81 ± 4.58	47.3± 5.41	60.56 ± 4.33	71.35 ± 3.83	n.d.
Glycosuria (mg/12h)	1.445 ± 0.142	0.817 ± 0.134	1.210 ± 0.148	0.859 ± 0.111	0.381 ± 0.064
Ca^{2+}/crea (mg/g)	197.3 ± 12.27	133.8 ± 10.51	221.4 ± 31.87	147.2 ± 23.47	64.7 ± 7.9
Calciuria (µmol/12h)	2.457 ± 0.251	1.823 ± 0.195	2.827 ± 0.333	2.348 ± 0.417	0.563 ± 0.118

n.d. not detectable

Figure 2.21: Summary of urinary parameters
Table showing mean values +/- s.e.m.

gests that the endocytic machinery is at least partially recovered upon BM transplantation. To confirm this hypothesis, we stained for megalin in kidney sections in ClC-5 KO mice that received GFP cells and mice that got KO cells. As expected, mice that received KO cells did not show any megalin staining. In contrast, mice that were transplanted with WT cells re-expressed megalin in their brush border membranes. It appeared that megalin expression was higher in regions of the kidney that showed more engraftment of GFP+ cells (Figure 2.23 A-D). The expression of megalin was further confirmed in western blots, where megalin protein expression in transplanted kidneys was intermediate to WT and KO mice that displayed normal or very low expression, respectively (Figure 2.23 E). In contrast, aquaporine 1 and Rab5, which are not affected by loss of ClC-5, remained unchanged in all treatment groups (Figure 2.23 E). Importantly, Clcn5 gene expression increased significantly in treated animals, and the expression level significantly inversely correlated with urinary loss of CC16 (Figure 2.23 F, G).

Engrafted cells are mononuclear phagocytes

To closer investigate the mechanisms that lead to the observed improvement in Fanconi syndrome, we analysed the location and identity of the transplanted cells in the kidney. Besides the fact that more GFP+ cells engrafted to the kidneys of ClC-5 KO mice than WT mice (Figure 2.20), the cells showed also a slightly different distribution pattern. In KO mice, the transplanted cells associ-

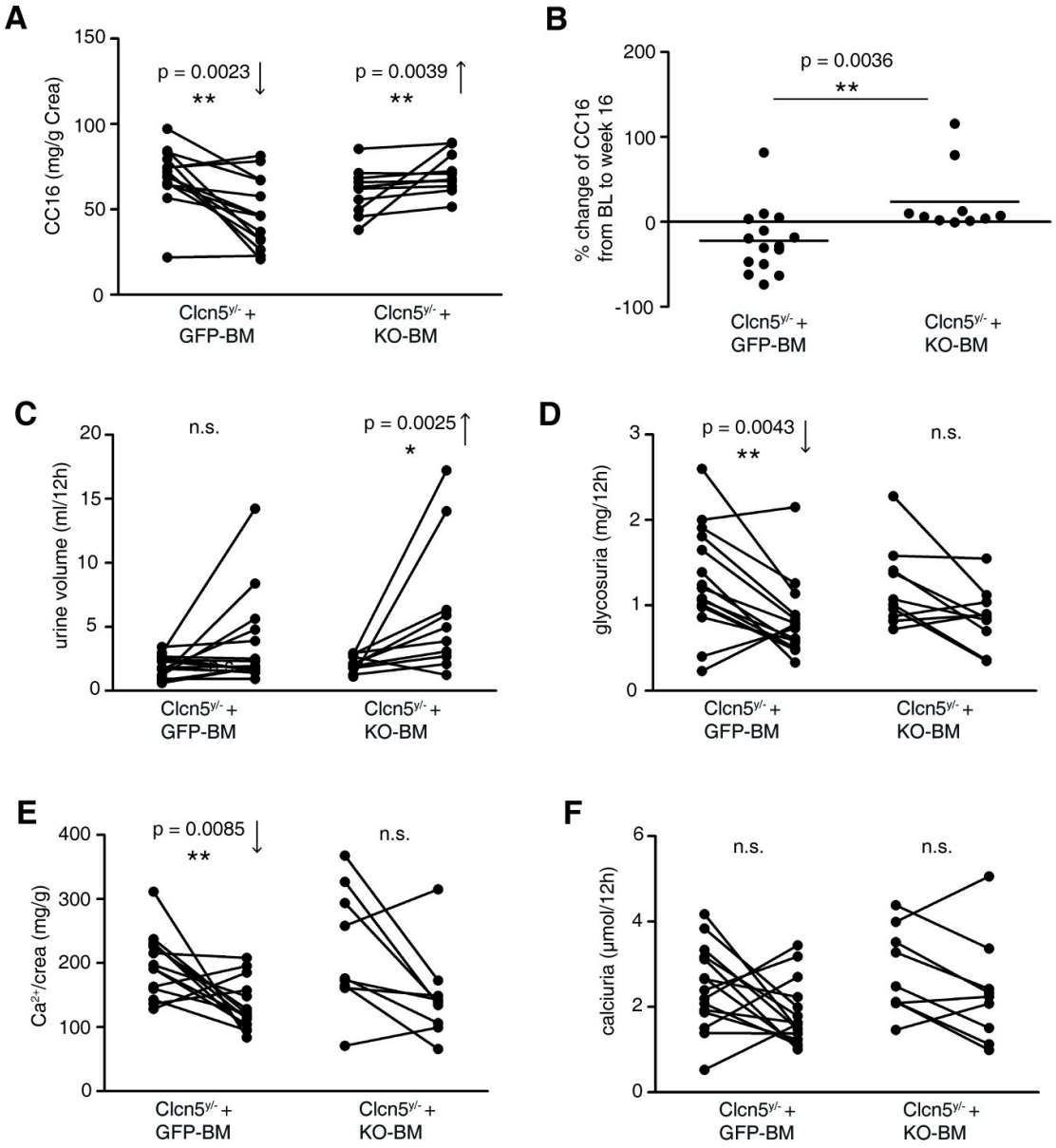

Figure 2.22: Urinary parameters of treated and control mice at BL and week 16
(A-F) Paired analysis of urinary parameters in ClC-5 KO mice treated with WT and KO cells. **(A)** CC16 (normalized to creatinine) **(B)** relative change in CC16 between measurements at BL and week 16, **(C)** total urine volume, **(D)** glycosuria, **(E)** Ca^{2+}/crea, **(F)** calciuria. Differences in urinary parameters at BL and week 16 were assessed with Wilcoxon signed rank test and relative change of CC16 between treatment groups with Mann-Whitney test.

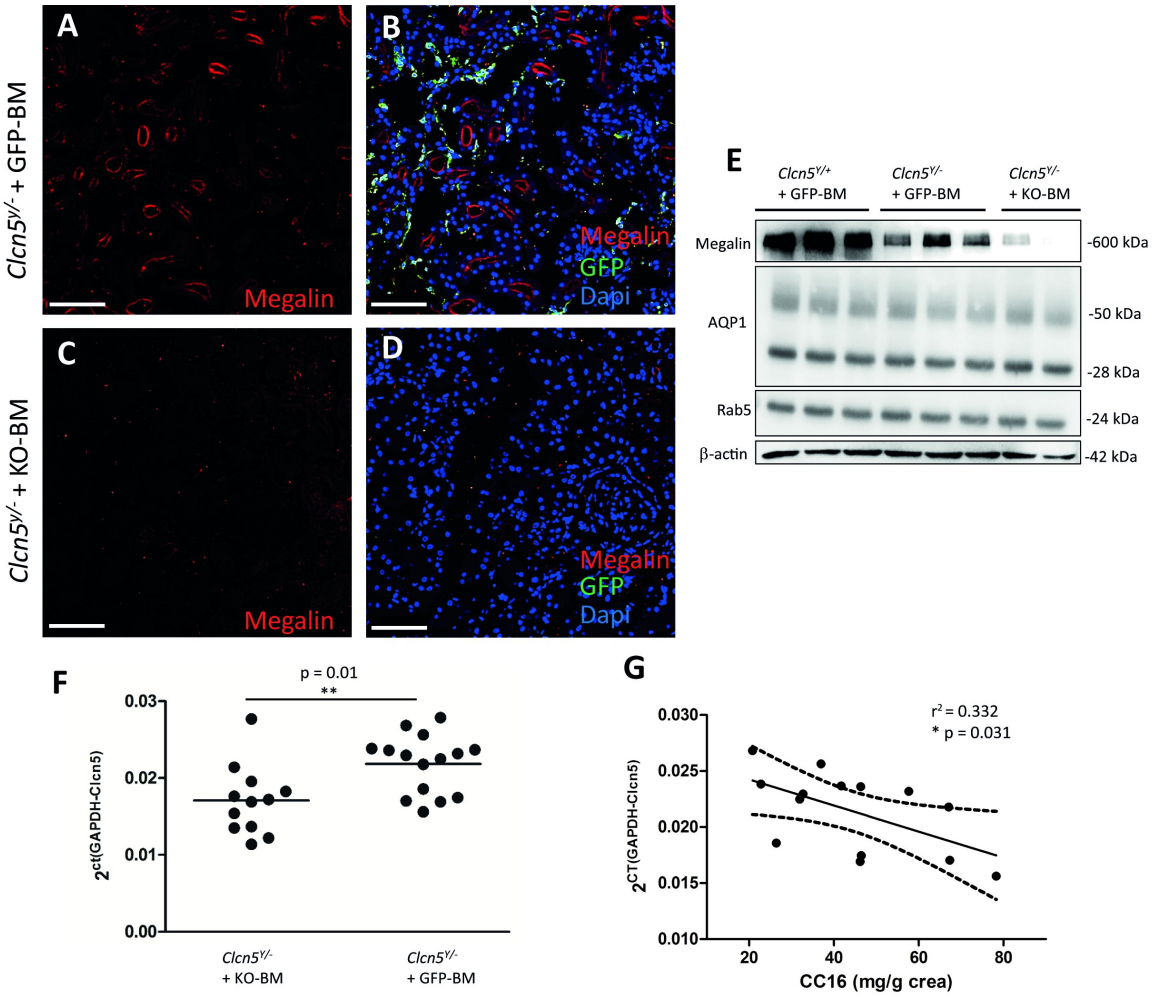

Figure 2.23: Recovery of megalin in brush border membranes of transplanted mice
Staining for megalin (red), GFP (green) and Dapi in mice transplanted with **(A, B)** GFP- or **(C, D)** KO-BM cells. **(E)** western blot for megalin, aquaporine 1 (AQP1) and Rab5 in kidney lysates. **(F)** mRNA expression of Clcn5 and **(G)** correlation between Clcn5 mRNA and urinary CC16. White bars correspond to 75 µm. Mann-Whitney test was used to assess difference between treatment groups, and correlation represents a linear regression-model with 95% CI.

ated closely to the tubular epithelial cells and their processes seemed to frame the tubuli, whereas in WT mice the transplanted cells sat in the interstitial space without such close contact with the tubuli (Figure 2.24; quantification in progress).

It is highly unlikely that hematopoietic cells transdifferentiate into non-hematopoietic cells. Accordingly, we did not observe any tubular epithelial cells that expressed GFP, all transplanted cells were found in the tubulointerstitial space. It might be possible that mesenchymal stromal cells (MSCs), that were present in the BM cell suspension upon transplantation, were recruited to the kidneys. Such cells do not migrate into healthy tissues, but upon irradiation injury, increased recruitment into peripheral organs has been observed [316]. In addition, MSCs have been shown to be renotropic in situations of acute injury and they may persist in kidneys for a long time [173, 317]. For these reasons, the engrafted cells might theoretically derive from the MSC lineage. However by forming a network of processes, the GFP-expressing cells phenotypically resembled rather dendritic cells (DCs) or macrophages than MSC-derived cells, which would be spindle shaped and compact. In the kidney, it is not possible to discriminate DCs and macrophages based on the expression of surface markers that are commonly used in lymphoid organs [284, 285, 318]. As DCs and macrophages further possess overlapping functions in the kidney, these cells are often referred to as „mononuclear phagocytes“ [284]. We confirmed this identity by staining the kidney sections for CD11c and F4/80 (Figure 2.25 A-F). Co-staining of these markers with GFP allowed the conclusion that these cells originated from the hematopoietic lineage. To further confirm this finding, we stained the slides with the fibroblast marker α-SMA. Indeed, there was close association between transplanted cells and fibroblasts, but no overlap of the stainings (Figure 2.25 G, H).

Discussion

For some conditions, such as diseases of the hematopoietic system (e.g. sickle cell disease or SCID), the potential of BM transplantation is evident, but it has also been successful in certain types of lysosomal storage diseases (LSDs). LSDs are a group of diseases that are characterized by enzyme deficiency in lysosomes, resulting in substrate accumulation, subsequent cellular dysfunction and apoptosis [319]. Here, healthy cells deriving from the transplanted hematopoietic system can engraft into various organ structures and through the close proximity with diseased

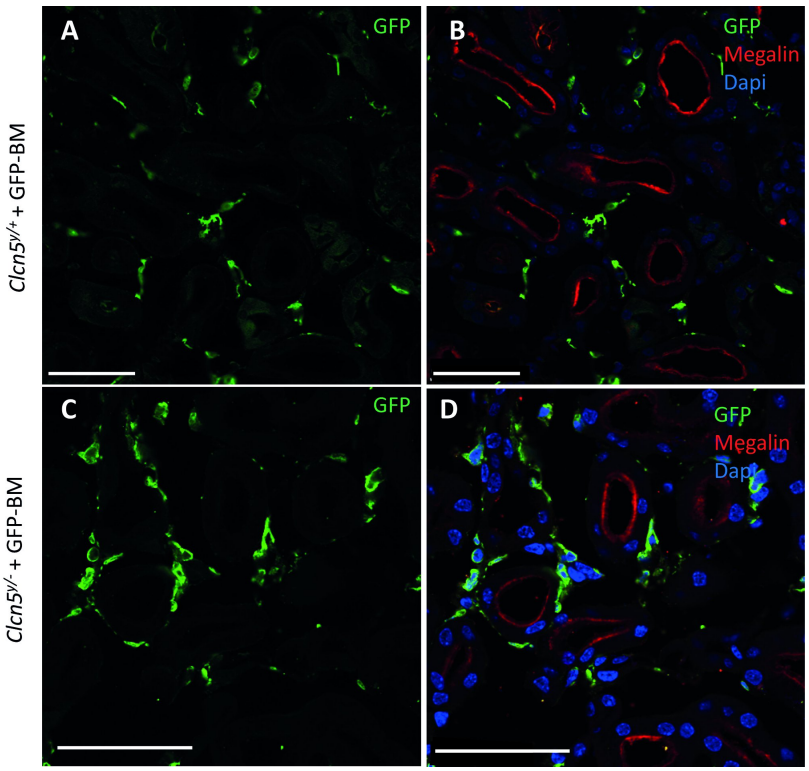

Figure 2.24: Distinct distribution pattern of transplanted cells
Staining for megalin (red), GFP (green) and Dapi. **(A, B)** WT mice and **(C, D)** ClC-5 KO mice transplanted with GFP+ cells. White bars correspond to 50 µm.

cells, they can replace defective enzymes by secretion. In Hurler Syndrome for example, HSC transplantation is being performed in patients for more than 30 years [320]. Cystinosis is also a LSD, but it is caused by defects in a membrane-bound transporter, circumventing the secretion and uptake of the functional protein. In this disease, the main positive effect of transplantation was the reduction of cysteine crystals in tissues, while improvement of kidney and serum parameters was only modest [212, 315]. The positive effects can possibly be attributed to phagocytic cells that engrafted in great numbers to the kidneys.

In the present study, we aimed to extend the finding that BM transplantation can improve diseases that are caused by membrane-bound transporters. In contrast to cystinosis, in Dent's disease we observed a significant improvement of renal Fanconi syndrome, as manifested most prominently in a decreased loss of CC16 in the urine. Engrafted cells seemed to belong to the family of mononuclear phagocytes based on their co-expression of F4/80 and CD11c. These cells are found in the tubulointerstitium and are important for tissue homeostasis, e.g. by

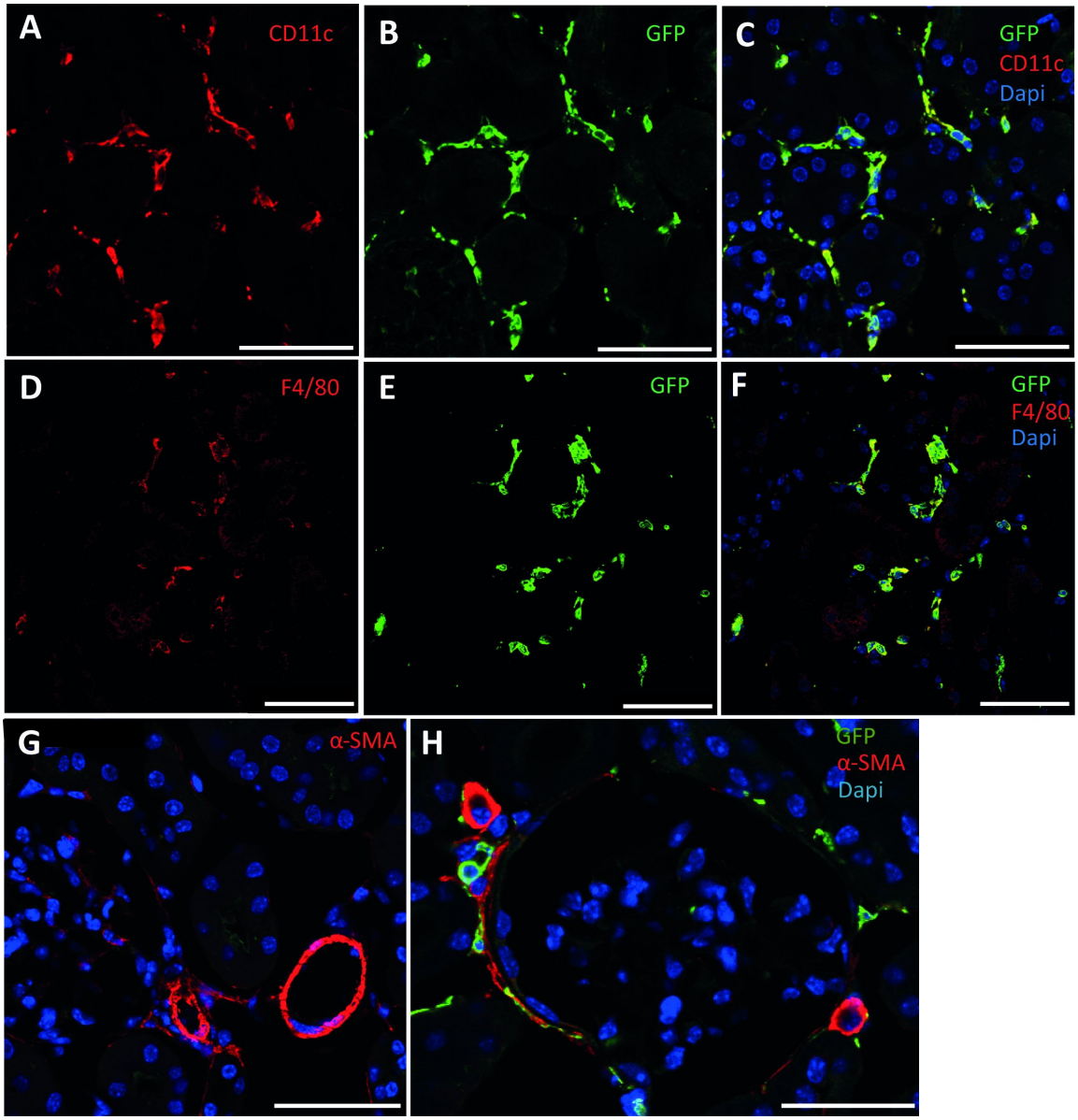

Figure 2.25: Transplanted cells are mononuclear phagocytes
Staining for GFP (green) and Dapi in ClC-5 KO mice transplanted with GFP cells and additional **(A-C)** CD11c (red) and **(D-F)** F4/80 staining. **(G)** α-SMA staining (red) in ClC-5 KO cells transplanted with KO cells, showing a vessel. **(H)** α-SMA (red), GFP (green) and Dapi in a kidney transplanted with GFP+ cells. White bars correspond to 50 µm.

maintaining tolerance to renal antigens and LMW proteins that are filtered, or by clearing apoptotic cells [284]. Surprisingly, the presence of functional mononuclear phagocytes resulted in a partial rescue of megalin expression in the brush border membrane of PT cells. This indicates that the process of receptor-mediated endocytosis might be functional to a certain level, explaining the decreased loss of CC16 in the urine. However at the moment, it can only be speculated about how this rescue of megalin is achieved.

Basically, two possible explanations could be envisaged: (1) There is no genetic defect of megalin in ClC-5 KO mice, but rather a defect at the cellular level due to the defective endosome/lysosome pathway. Thus, the rescue of megalin could be explained by a constant protein transfer of this receptor from mononuclear phagocytes to epithelial cells. However, this is rather unlikely, as functional megalin has not been shown to be expressed in such phagocytes. In fact, macrophages in the liver constitutively express megalin mRNA, but on protein level only the megalin C-terminal fragment is present [321]. (2) More reasonable might be the indirect rescue of megalin by transferring a certain amount of functional ClC-5 proteins or mRNA to epithelial cells. It has been shown that peritoneal macrophages, but not peripheral blood monocytes, express high levels of ClC-5 protein [322]. Whether DCs express ClC-5 has not been investigated. It is well possible that the transplanted cells at least partially express ClC-5 when acquiring their tissue-resident phenotype. This might explain the significant increase of Clcn5 mRNA in kidneys of mice transplanted with WT BM compared to KO controls (Figure 2.23 E). Based on the close proximity of transplanted cells with epithelial cells, one might speculate that transfer of Clcn5 mRNA or protein between these cells takes place. In fact, transfer of extracellular vesicles (EV) is considered to be a common way of intercellular communication between neighbouring cells [323–325]. Low-level expression of functional ClC-5 transporter in epithelial cells then may prevent complete loss of megalin, restoring the endocytosis machinery to a certain degree. Indeed, Clcn5 gene expression kidneys of treated animals significantly inversely correlated with CC16 loss in the same mouse, supporting this hypothesis. However, more research will be needed to get a clearer picture of these fascinating and potentially important findings.

Contribution of S.S.G. to the manuscript

Helped planning and organizing the experiments, performed all in vivo experiments and some stainings, analysed the data and wrote the manuscript.

3 Discussion

Apoptosis is of overriding importance for the proper functioning of the immune system. In particular, the intrinsic apoptosis pathway is involved in processes such as the elimination of self-reactive thymocytes, selection of high affinity clones and contraction of the Teff pool after antigenic clearance through the dynamic expression of pro- and anti-apoptotic factors. With the advent of BH3 mimetics that allow to specifically target certain anti-apoptotic factors, and a detailed understanding of apoptosis regulation in the immune system, previously unrecognised therapeutic options can be envisaged. This novel class of drugs could find application in diseases/conditions that are characterized by unwanted or overshooting immune responses.

Immunosuppressive action of ABT-737

Our group has previously found the Bcl-2, Bcl-xL and Bcl-w inhibitor ABT-737 to suppress allogenic T cell responses in vitro in a concentration-dependent manner [244]. Short-term treatment of naïve animals with ABT-737 resulted in a marked reduction of platelets and lymphocytes in the spleen, thymus and the mucosa-associated lymphatic tissue, while non-lymphoid tissues such as the kidney, liver and intestinal epithelial cells did show no signs of increased apoptosis [244]. Thus, the action of ABT-737 seemed to be confined to cells of hematopoietic origin. Interestingly, while naïve cells were efficiently killed by ABT-737, freshly activated T cells were refractory to apoptosis induction [61]. TCR engagement leads to release of calcium from intracellular stores and calcineurin activation, which subsequently dephosphorylates NFAT. Activated NFAT cooperates with other transcription factors that are activated upon TCR or co-stimulatory receptor signalling, to induce the expression of genes that are required for the differentiation of naïve T cells into fully activated cells [326]. One of the many genes that are activated by NFAT is anti-apoptotic A1, which was responsible for the observed ABT-737 resistance in activated T cells. Accordingly, this resistance could be overcome by administration of the calcineurin inhibitor CsA at the time of antigen encounter [61]. Interestingly, also in naïve cells, CsA and ABT-737 seemed to have a synergistic effect in apoptosis induction, as CsA reduced Bcl-2 expression and thereby lowered the IC50 value of ABT-737. In vivo, this fact was manifested by significantly lower lymphocyte counts upon com-

bined drug administration.

Based on the growing understanding of the effects of ABT-737 on immune cells, several approaches to actually manipulate the immune system could be envisaged. When the drug was administered under naïve conditions (which is before antigen encounter or inflammation), it depleted lymphocytes and thereby reduced the precursor frequency of potentially „dangerous“ cells. This immunosuppressive effect of ABT-737 was illustrated for example in diverse models of autoimmunity (SLE, collagen-induced arthritis, delayed-type hypersensitivity). ABT-737 reduced the severity of these diseases when the treatment regimen was prophylactic or started at the very first manifestations of the disease [327]. In a mouse model of spontaneous colitis, ABT-737 induced lymphopenia went along with reduced systemic TNF and lL-1β gene expression, and decreased inflammation in the intestine [328]. Similar results were obtained with diabetic mice that were treated with ABT-737 for 5 days and then transplanted with an allogenic pancreatic islet graft. ABT-737 treatment prevented the rejection of this low-immunogenic graft, resulting in lower blood glucose levels [241]. In contrast, the immunosuppressive effect of ABT-737 (with or without CsA) was not sufficient to prolong survival of highly immunogenic fully MHC-mismatched skin grafts, even when treatment was started 5 days before transplantation and continued until rejection. Nevertheless, treatment resulted in a significant reduction of allospecific IgG and IgM antibodies in the serum [244]. Rejection of MHC-I single antigen mismatched skin grafts could be prevented in about 50% of animals when they received combined ABT-737 and CsA treatment, while both drugs as single agent did not have any effect [244].

Taken together, ABT-737 has proven to display an immunosuppressive effect in vivo through clone size reduction without affecting the functionality of remaining lymphocytes. AbbVie, the company that produces ABT-737 and its oral analogue ABT-263, has recently completed a clinical trial with ABT-199 (a Bcl-2 only inhibitor not leading to thrombocytopenia, a side effect of ABT-263) in SLE patients, presumably with the purpose to reduce lymphocyte numbers and thus profit from its immunosuppressive action.

Differences in apoptosis among cell populations

Having observed the general reduction of lymphocytes upon ABT-737, we aimed at closer investigating apoptosis regulation in T cell subsets that are particularly important in the transplantation field; Tm and Tregs. Preformed Tm are involved in graft rejection and preclude clinical translation of successful tolerance strategies in rodents. We have shown that during the transition of activated T cells to the memory stage, the relative expression of anti-apoptotic factors changes. The resistance factor A1 was strongly down regulated in Tcm and to a lesser extent in Tem, while the importance of Bcl-2 for survival seemed to increase. These dynamic changes were reflected in the susceptibility to ABT-737 induced apoptosis. Tm were considerably more sensitive to apoptosis than early-activated T cells [300]. Moreover, when applied at time of transplantation, ABT-737 could reverse the accelerated rejection mediated pre-existing allo-specific Tm, leading to similar rejection kinetics as observed in naïve mice [300].

Furthermore, we compared Bcl-2 family protein levels among FoxP3+ Tregs and naïve CD4 Teffs. When unstimulated, Treg expressed slightly more Bcl-xL protein, while the other factors were similar among the two cell populations. Nevertheless, Tregs displayed approximately 10x higher resistance to ABT-737 induced apoptosis in vitro. When stimulated with IL-2, Treg resistance increased significantly due to strong upregulation of Mcl-1 and A1. Our findings are consistent with a recent study that identified Mcl-1 as the predominant survival factor in Tregs. There, genetic deletion of Mcl-1 resulted in specific loss of Tregs among the CD4 population [70]. In vivo application of ABT-737 to naïve mice resulted in lymphopenia, but importantly, the proportion of Tregs among the CD4 population increased, leading to a change in the balance of Tregs versus Teffs.

In addition to these two examples of differential apoptosis regulation among different T cell populations, it has recently been reported that plasmacytoid and conventional dendritic cells (pDC and cDC) present a fundamentally distinct expression pattern of anti-apoptotic Bcl-2 family members. Bcl-2 and Bcl-w are highly expressed in pDC, while A1 is only expressed in cDCs [329]. Thus, cDC were highly resistant towards Bcl-2 blockade with ABT-199 in vitro, and in vivo application of ABT-199 resulted in selective depletion of pDC while cDC numbers were maintained [329].

These examples clearly illustrate that dif-

ferent subpopulations may depend on different survival factors. In many pathologic conditions, certain cell populations are of predominant importance and specific targeting of these cells would be useful. With the ever-growing repertoire of Bcl-2 inhibitors, the possibilities to eliminate a specific population from the immune repertoire are manifold. As such, BH3 mimetics may not only be a novel class of drugs for general immunosuppression as it has been suggested, but rather a high-precision tool to specifically target cells. However, in order to fully profit from the therapeutic options offered by BH3 mimetics, our understanding of the regulation of the intrinsic apoptosis pathway in cell subpopulations and under certain disease states needs to grow considerably. Especially also in human cells, where very little is known to date. We tested the effect of ABT-737 on peripheral blood lymphocytes to see whether we can achieve a similar in vitro enrichment of Tregs as in mouse cells, but unfortunately, this was not the case.

Exploit the growing understanding of lymphocyte apoptosis for novel immunomodulatory therapies

This knowledge eventually allowed us to exploit the immunomodulatory properties of ABT-737 in more sophisticated tolerance protocols. The extent of Tm reduction was sufficient to overcome the barrier they pose to tolerance induction. Donor skin grafts were rejected faster than control grafts in mice containing donor-reactive Tm when they were treated with DST and CD154/CD40 blockade, showing that the tolerance protocol did not have any effect in primed mice. In contrast, a short course of ABT-737 in addition to DST completely reversed the picture and resulted in profound donor-specific hyporesponsiveness [300]. This finding suggested that not only the Tm barrier is overcome with ABT-737 treatment, but that further mechanisms come into play.

DST/costimulation blockade-induced hyporesponsiveness has been shown to be associated with diverse mechanisms, such as T cell anergy, deletion and regulation by Treg cells [279, 330, 331]. Costimulation is not only needed for T cell proliferation and cytokine production, but also for prolonged survival by inducing Bcl-xL expression [191, 332]. On the other hand, Bim is induced in response to TCR engagement, and costimulation blockade further increases Bim expression [191]. Thus, costimulation blockade reduces viability of allo-activated CD8 T cells by reducing Bcl-xL and increasing Bim expression, and it seems that this process of clonal deletion under CD154 blockade could

be further potentiated by Bcl-xL blockade with ABT-737 [191]. Regarding regulation, Tregs are required for maintenance of peripheral tolerance towards allo-antigens. Tregs develop de novo upon antigen-encounter under CD154 blockade, and CTLA-4 is required for long term survival of skin grafts under these conditions [134, 279]. We have shown that Tregs are extremely resistant towards ABT-737 treatment in such an environment. Thus, addition of ABT-737 to the established DST protocol resulted in (1) more efficient deletion of allo-reactive T cells and (2) at the same time boosted Treg-mediated suppression. This led to an impressive donor-specific hyporesponsiveness in a strain combination where DST plus CD154 antibodies otherwise was not sufficient to prolong skin graft survival.

GVHD has been characterized as an „imbalance between the effector and regulatory arms of the immune system“ [333] . When Tregs are depleted from the transplant inoculum, GVHD worsens, while adoptive transfer of Tregs ameliorated the disease [334]. Treatment of transplant recipients with ABT-737 and low-dose CsA is reasonable, as it reduces lymphocyte numbers while Tregs are spared, thus the balance between Tregs and Teffs is restored. In combination, these two drugs act as immunosuppressants and allow eliminating also TCR stimulated Teffs. The possibility to establish mixed chimerism and allo-tolerance with a short induction protocol consisting of ABT-737, low-dose CsA and costimulation blockade without myelosuppression is remarkable [191]. Here, all the above discussed aspects of ABT-737 mediated immune-regulation act in concert.

Potential applications of Bcl-2/Bcl-xL inhibition in clinical transplantation

The beneficial effects that we have observed in our experimental research with ABT-737 should at least partially be confirmed in human cells before clinical translation of our concepts could be envisaged. Important in this regard would be the confirmation of Tm sensitivity to Bcl-2/Bcl-xL inhibition. If this is the case, the most promising and widespread application of ABT-737 (or the bioavailable analogue navitoclax) would be the induction therapy in highly sensitized organ recipients. Treatment is expected to result in clone size reduction of Tm, lymphopenia and potentially a tolerogenic environment due to large amounts of apoptotic bodies. Importantly, lymphopenia is reversible within a few days after discontinuation of treatment. This represents a huge advantage compared to other T cell depleting agents in clinical practice like ATG, which

induce sustained, and in the case of an infection potentially dangerous, lymphopenia.

Regarding tolerance induction, plans exist to combine navitoclax with established tolerance protocols in non-human primates. This will prove whether navitoclax displays similar tolerogenic properties in higher animals as ABT-737 in mice, and whether it also supports engraftment of HSCs. If this is the case, preparatory irradiation of HSC recipients might be reduced or replaced. If successful, these studies could pave the way for addition of navitoclax to established tolerance induction protocols in kidney transplant recipients in the future [194, 196].

Another potential application field for Bcl-2/Bcl-xL inhibition might be adoptive T cell therapy. TCR-stimulated T cells upregulate A1 and thereby become strongly resistant towards ABT-737 induced apoptosis [61]. This fact could be exploited by stimulating a patient's T cells in vitro with the antigen of interest (e.g. a tumor or viral antigen). Subsequent incubation with ABT-737 would easily allow to specifically select antigen-specific T cells in the culture. These cells could then be expanded further and transferred back to the patient, for example upon cytomegalovirus infection post-transplantation.

The therapeutic potential of BM transplantation in treating chronic kidney diseases

There have been numerous studies over the past 15 years that tested the effectiveness of BM cells as therapy in a wide variety of conditions affecting the kidneys. And there has been considerable controversy about the potency of this treatment approach. In that regard, it is important to note that it is difficult to directly compare acute disease models with chronic progressive models, and HSCs with MSCs [308].

BM-derived stem cells have been shown to engraft to the kidneys and help improve renal function, in both, acute and chronic kidney diseases. For example, these cells were shown to differentiate into mesangial cells in a Thy1.1 nephritis model in rats [335], and systemic infusion of resulted in improved kidney function after acute kidney injury [336]. Similarly, human HSCs were shown to have beneficial effects in kidney I/R injury in mice when infused systemically. These cells were recruited to the kidney parenchyma, peritubular capillaries and a few to the perivascular space. This recruitment was associated with increased repair of the microvasculature and tubular epithelial cells, leading to reduced mortality and improved kidney function [337]. And as discussed in detail in the

introduction, in a pre-clinical model of cystinosis BM transplantation resulted in engraftment healthy hematopoietic lineage cells to the kidney, which was accompanied by improved kidney function [212]. In contrast, protection through paracrine effects (e.g. the release of large amounts of VEGF) was attributed to MSCs [208, 338]. Taken together, kidney repair by stem cells is a complex process, and it is difficult to draw conclusions about the therapeutic benefit of this therapeutic approach at this stage.

When discussing the potential of stem cell therapy for kidney diseases, it is not only important to discriminate between acute and chronic conditions and the stem cell type used, but it is also essential to discuss the mode of treatment. It is comprehensible that systemic infusion of BM-derived cells around the time of injury can have immediate protective effects. However, when looking at chronic conditions that are caused by genetic defects, such as cystinosis or Dent's disease, such short-term treatment approaches cannot be sufficient to improve kidney function in the long-term. Indeed, the group that investigated the cystinosis model has explicitly compared the therapeutic effect of unfractionated BM, isolated HSCs and MSCs, and they found only temporary improvement of renal function with MSC infusion [212]. In my opinion, a source of healthy cells without the genetic defect needs to be available for continuous recruitment to the defective organ. And the only possibility to establish such a pool of cells is the transplantation of HSCs, which can give rise to all types of blood cells such as T and B lymphocytes, eosinophils, neutrophils, basophils, DCs and monocytes/macrophages. Importantly, these cells are not stationary, but they circulate through the body and thus can get in contact with the diseased organ, which is a pre-requisite for engraftment. Once recruited to the kidney, it is still elusive how these cells can restore functional defects. The previously suggested transdifferentiation into/or fusion with the target cells (in our case proximal epithelial cells) is questionable.

The tubulointerstitium contains many different BM-derived cells. It has been shown that mostly CD45 expressing cells occupy the peritubular interstitial space in the cortex of rat kidneys [339]. Macrophages are tissue-resident phagocytic cells that long have been considered to be mainly immune cells and their presence was thought to be deleterious and equalled to „inflammation". More recent research however, has revealed their involvement in tissue homeostasis and repair, regeneration and resolution of inflammation

[340, 341]. For example, a study has shown that M2-polarised macrophages actively promoted repair of tubules and angiogenesis [342]. In cystinosis, WT macrophages have been shown to form tunnelling nanotubes to diseased neighbouring cells, which might allow the exchange of cystinosin-bearing lysosomes [343]. However, the proposed mechanism still remains somewhat obscure. DCs are another tissue-resident, highly heterogenous hematopoietic lineage-derived cell population that resides in the tubulointerstitium of the renal cortex. They form long cytoplasmic processes that extend between and around tubules [339, 344]. In addition it has been suggested that renal DCs can directly interact with renal cells such as epithelial cells and podocytes [285]. It is important to note, that most research that was aimed at elucidating the role of „macrophages“ or „DCs“ in the kidney, was performed by either looking at F4/80 or CD11c expressing cells. However, it is now evident that the classification of tissue-resident phagocytes in the kidney is more complex. Several distinct phagocytic subpopulations have been described [345]. These cells show overlapping classical „macrophage- or DC-functions“ in vivo and most of them co-express CD11c, F4/80 and MHCII [284]. Thus, markers that allow discriminating macrophages and DCs in lymphoid organs, are less useful in non-lymphoid tissues such as the kidney. For these reasons, talking about mononuclear phagocytes is more appropriate.

In our study of Dent's disease, we observed a partial rescue of megalin in the brush border membranes of PT cells of transplanted mice, which might explain the reduced loss of CC16 in the urine. CC16 is a cubilin ligand, a receptor that is co-expressed with megalin and is also lost in Dent's disease. As shortly discussed in the experimental part, it has been shown that peritoneal macrophages highly express ClC-5, while monocytes do not [322]. This suggests that tissue-resident phagocytes in the kidney might also express ClC-5. This possibility is supported by the fact that Clcn5 gene expression significantly increased in KO mice that were transplanted with WT cells. I believe that transfer of functional protein and/or Clcn5 mRNA via extracellular vesicles (EVs) is taking place between the closely entangled mononuclear phagocytes and the epithelial cells. In fact, exchange of EVs has been suggested to be the third mode of communication between neighbouring cells, besides direct cell-cell contact and the secretion of molecules [323]. As direct investigation of this process in vivo is challenging, most research on EVs has been undertaken in vitro. However, very recently

an in vivo study has shown that aggressive tumor cells transfer mRNA to less malignant cells via EVs, which in turn also become more malignant [324]. DCs and macrophages both constitutively secrete EVs in vitro, thus it is well possible that material from these cells is transferred to epithelial cells in our model [346, 347].

Is clinical translation of HSC transplantation an achievable goal for diseases such as Dent's?

When considering HSC transplantation as potential therapy in non-malignant conditions, the pros and cons have to be balanced carefully. The more devastating a disease, the more severe side effects deriving from HSC transplantation are acceptable. Dent's disease is certainly less severe than cystinosis or some other LSDs, where HSC transplantation is being performed already. For this reason, for HSC transplantation to be a real treatment option for diseases such as Dent's, the toxicity of conditioning regimen of recipients needs to be reduced significantly. Effort is given at developing such protocols for mixed chimerism induction for transplant recipients, and for HLA-matched HSC recipients, the Stanford protocol is quite successful already [194]. However, more than half of the patients displayed only transient chimerism that was lost within one year. Interestingly, this loss of chimerism did not affect tolerance towards the transplanted kidney, a phenomenon called „split tolerance“ [194]. When considering to use such a reduced conditioning approach for a renal disease where continuous replenishment of interstitial cells by BM-derived cells is presumably needed, such a loss of chimerism might pose a problem. For example, turnover of renal DCs is only about 14 days under homeostatic conditions, thus persistent chimerism is needed [348]. On the other hand, full chimerism is less desirable due to an increased risk of GVHD.

In order to answer the question whether HSC transplantation will be used in clinics to treat diseases like Dent's in the future, many more studies will be needed. Clinical protocols for HSC transplantation have to be advanced to further reduce toxicity of conditioning. At the same time, mechanisms that lead to loss of chimerism in patients need to be understood and possibly prevented, for example using DST . Furthermore, with the help of pre-clinical models, studies aimed at understanding the mechanisms of improvement of diseases like Dent's or cystinosis have to be undertaken. Importantly, BM transplantation has to be performed also in allogeneic strain combinations and with protocols that induce mixed chimerism. Here, it will be important to correlate the chimerism

level with the outcome of the disease to see whether mixed chimerism is sufficient for this purpose.

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Regular Article

TRANSPLANTATION

Targeting apoptosis to induce stable mixed hematopoietic chimerism and long-term allograft survival without myelosuppressive conditioning in mice

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Key Points

- Immunological tolerance can be achieved by direct modulation of the intrinsic apoptosis pathway in peripheral lymphocytes.

Induction of mixed hematopoietic chimerism results in donor-specific immunological tolerance by apoptosis-mediated deletion of donor-reactive lymphocytes. A broad clinical application of this approach is currently hampered by limited predictability and toxicity of the available conditioning protocols. We developed a new therapeutic approach to induce mixed chimerism and tolerance by a direct pharmacological modulation of the intrinsic apoptosis pathway in peripheral T cells. The proapoptotic small-molecule Bcl-2 inhibitor ABT-737 promoted mixed chimerism induction and reversed the antitolerogenic effect of calcineurin inhibitors by boosting the critical role of the proapoptotic Bcl-2 factor Bim. A short conditioning protocol with ABT-737 in combination with costimulation blockade and low-dose cyclosporine A resulted in a complete deletion of peripheral donor-reactive lymphocytes and was sufficient to induce mixed chimerism and robust systemic tolerance across full major histocompatibility complex barriers, without myelosuppression and by using moderate doses of bone marrow cells. Thus, immunological tolerance can be achieved by direct modulation of the intrinsic apoptosis pathway in peripheral lymphocytes—a new approach to translate immunological tolerance into clinically applicable protocols. (*Blood*. 2013;122(9):1669-1677)

Introduction

Induction of allograft tolerance, a state in which the immune system accepts donor organs but normally responds to foreign antigens, represents the ideal solution for preventing rejection after solid-organ transplantation without immunosuppression-related toxicity. Among the different experimental models to induce tolerance, very few were successful in large animals and only one strategy—namely, the induction of mixed chimerism by combined transplantation of a solid organ and hematopoietic stem cells from the same donor—was successful in clinical pilot studies.[1]

The main mechanism of tolerance in mixed chimeras is central deletion of newly arising donor-reactive lymphocytes induced by the presence of donor-derived antigen-presenting cells in central lymphatic organs.[2,3] Preexisting mature donor-reactive T cells are not exposed to this tolerization process and need to be removed from the T-cell repertoire by a conditioning therapy to prevent rejection. In the first clinical mixed chimerism studies, peripheral T-cell tolerization was obtained by unselective lymphocyte depletion through irradiation or profound T-cell depletion by antibodies.[4-7] This approach was effective, but inevitably induced pancytopenia and with some protocols also engraftment syndrome. Moreover, in nonhuman primates and patients, hematopoietic chimerism was not always stable over time, and in some protocols chimerism was only transiently detectable (apparently without precluding tolerance maintenance).[4,5] Other groups reported the establishment of full chimerism, a condition that, however, bears a high risk for graft-vs-host disease.[6]

The hope for a selective and less toxic option to clonally delete peripheral donor-reactive T cells was provided by the development of immunomodulatory strategies based on costimulation blockade. Blocking CD28/CD80/CD86 and CD154/CD40 signaling induces anergy and deletional tolerance by activation of the apoptosis cascade in alloreactive T cells.[3,8,9] However, this strategy displayed a reduced efficacy in combination with standard immunosuppression by calcineurin inhibitors[10,11] and was not sufficient to induce tolerance in case of a high donor-reactive T-cell precursor frequency.[12]

Resistance to anti-CD154-induced or CTLA4Ig-induced tolerance in mice overexpressing the antiapoptotic factor Bcl-xL indicates that the regulation of the intrinsic apoptosis pathway by the Bcl-2 family is pivotal in this setting.[13,14] The recent advent of selective Bcl-2 family inhibitors offers new pharmacological options to modulate these physiological processes. Of particular clinical interest are the small molecules ABT-737 and ABT-263 (navitoclax),[15,16] rationally designed molecules with antineoplastic[17] and immunomodulatory properties.[18-20] ABT-737 acts as a "sensitizer BH3-only protein": it inhibits the antiapoptotic Bcl-2 factors Bcl-2, Bcl-xL, and Bcl-w and enhances the effect of proapoptotic endogenous "activator BH3-only proteins," such as

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Bid or Bim.[15] ABT-737 has a selective proapoptotic activity on peripheral lymphocytes and platelets, but does not induce myelosuppression, as shown by the unaffected numbers of granulocytes and erythrocytes in peripheral blood.[18,20] This activity profile resulted in a favorable therapeutic index for ABT-263 in first clinical cancer trials.[21] Moreover, ABT-737 inhibited allogeneic immune responses through apoptosis induction in donor-reactive T cells.[20] This effect was markedly increased in combination with cyclosporine A (CsA), because calcineurin inhibition prevented resistance of activated T cells to ABT-737 in the first days after antigen recognition by blocking the expression of the antiapoptotic factor Bcl-2A1, which is not antagonized by ABT-737.[22] Furthermore, CsA reduced the expression of Bcl-2 in lymphocytes and therefore increased the proapoptotic effect of ABT-737 without affecting its selectivity profile.[23]

In this study we evaluated Bcl-2 inhibition as a novel option to induce donor-specific tolerance in the context of mixed chimerism protocols. We found that Bcl-2 inhibition promoted tolerance by supporting the deletion of donor reactive T cells in combination with costimulation blockade. The tolerogenic effect of ABT-737 on peripheral lymphocytes was mediated by a pharmacological boosting of the proapoptotic factor Bim, which was identified as a critical player for establishment of mixed chimerism. By targeting the intrinsic apoptosis pathway, we developed a novel protocol to achieve complete deletion of donor-reactive T cells with a minimal toxicity and to induce robust systemic tolerance without myelosuppression,

Methods

Mice

C57BL/6 (B6, H-2^b), CBA (H-2^k), BALB/c (H-2^d), BM3.3 (CBA, H-2^k), and Bim knock-out mice (Bim$^{-/-}$, H-2^b) were housed in specific pathogen-free conditions at the University of Zürich. The BM3.3 mouse[24]—which expresses on all CD8 T cells a transgenic T-cell receptor (TCR) selective for a naturally processed octapeptide bound to the allogeneic major histocompatibility complex (MHC) class I molecule H-$2K^b$—was kindly provided by A.-M. Schmitt-Verhulst.[25] Bim$^{-/-}$ mice were kindly provided by Andreas Strasser.[26] All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office, Canton of Zürich, Switzerland).

Conditioning and bone marrow (BM) procedures

Different conditioning protocols were tested as indicated. In general, B6-recipient mice received 1.5 or 3 Gy total body irradiation (TBI) from a ^{137}Caesium irradiator on day −1 with respect to bone marrow transplantation (BMT). Hamster anti-mouse CD154 (MR1, 2 mg; purchased from Bio-X-cell, West Lebanon, NH) was administered intraperitoneally (i.p.) 6 to 12 hours before transplantation with 25×10^6 fully MHC-mismatched CBA BM cells by tail vein injection. In some experiments, CTLA4Ig (abatacept, 0.5 mg; provided by Bristol-Myers Squibb, New York, NY) was administered at day 2 after BMT. ABT-737, provided by Abbott Bioresearch (Worcester, MA), was dissolved in polyethylene glycol, Tween 80, dextrose solution, and dimethylsulfoxide and injected intraperitoneally at 50 mg/kg. CsA (Sigma-Aldrich, Buchs, Switzerland) was dissolved in ethanol and cremaphor EL (Sigma-Aldrich), then diluted in phosphate-buffered saline and injected subcutaneously at 10 mg/kg. Both ABT-737 and CsA were administered daily from day −3 to day 12; on day −2 and day −1, mice received 2 injections for a total of 100 mg/kg of ABT-737 and 20 mg/kg of CsA. On day 0, ABT-737 and CsA were not administered. To monitor the deletion of donor-reactive CD8 T cells, we adoptively transferred 20×10^6 syngeneic BM3.3 splenocytes to CBA recipients before starting the conditioning protocol with B6 BM cells. The transgenic H-$2K^b$ reactive BM3.3 CD8 T cells were monitored over time in peripheral blood in a fluorescence-activated cell sorter (FACS) using the clonotypic antibody Ti98,[27] kindly provided by A.-M. Schmitt-Verhulst, and stained with a secondary phycoerythrin (PE) rat anti-mouse IgG, purchased from Becton Dickinson (Basel, Switzerland).

Skin grafting

Mice were shaved and anesthetized with ketamine/xylazine. Full thickness tail skin (about 1 cm^2) from CBA (BM donor) or BALB/c (3rd party) mice were grafted 3 to 6 weeks after BMT and considered rejected when <10% of the graft remained viable. In some experiments a second skin grafting was performed using the same procedure.

Flow cytometric analysis of chimerism and detection of allospecific antibodies

Flow cytometric (FACS) analyses were performed with a BD-FACSCanto (Becton Dickinson, Basel, Switzerland). Chimerism was analyzed in white blood cells at different time points after BMT, in spleen and thymus as indicated. Donor-derived cells were identified by fluorescein isothiocyanate (FITC)–conjugated anti-H-$2D^k$ or anti-H-$2K^k$ (Becton Dickinson). The cells were counterstained with anti-CD4-PE, anti-CD8-APC, anti-B220-PE, anti-CD11b-APC, anti-CD11c-APC, anti-CD49b-PE (for natural killer [NK] cells) antibodies purchased from eBioscience (Frankfurt, Germany). Background signal measured in a naïve B6 mouse was subtracted to determine the percentage of donor-derived cells. Allospecific antibodies were measured in indirect FACS: CBA, and Balb/c splenocytes were incubated with recipients' serum (1:5 dilution in FACS buffer) and subsequently stained with a secondary anti-mouse FITC-conjugated IgG antibody (eBioscience). Mean fluorescence intensity was determined in FACS gating on CD8-positive cells.

Mixed lymphocyte reaction (MLR)

MLR were performed in 96 wells plates with responder splenocytes stimulated by splenocytes from BM-donor, 3rd party, or syngeneic mice at a final concentration of 4×10^6 cells/mL in Roswell Park Memorial Institute medium containing 10% fetal bovine serum, penicillin 100 U/mL, streptomycin 100 μg/mL, and 2-mercaptoethanol 50 μM. T-cell proliferation was measured by incorporation of ^{3}H-thymidine (PerkinElmer, Waltham, MA) added to the culture on day 4 after stimulation. For the selective analysis of alloreactive CD8 T cells in vitro, BM3.3 spenocytes were stimulated with CD8 T-cell-depleted splenocytes from B6 (allogeneic) or CBA (syngeneic) mice and analyzed in FACS gating on CD8 T cells. After cell permeabilization, the level of different Bcl-2 factors in alloreactive CD8 T cells was detected in FACS, as previously described.[23] Bcl-2 was stained using an anti-mouse Bcl-2-PE from Becton Dickinson, Bcl-xL with an Alexa Fluor 488 conjugated antibody from Cell Signaling Technology (Danvers, MA). Mcl-1 was detected with a monoclonal rabbit anti-mouse Mcl-1 antibody from Abcam (Cambridge, United Kingdom), Bim with a polyclonal rabbit antibody detecting total Bim (EL, L, and S isoforms) purchased from Cell Signaling, and then stained with a FITC anti-rabbit IgG (eBioscience). Splenocytes were sorted by automatic magnetic cell separation using an autoMACS proseparator according to the protocols of Miltenyi Biotec (Bergisch Gladbach, Germany). For polyclonal stimulation of splenocytes, anti-CD3 and anti-CD28 antibodies were used (eBioscience). Cell viability was measured by propidium iodide exclusion in FACS.

Statistics

Student *t* test was used to compare values between groups. A P value $< .05$ was considered significant. Graph Pad Prism Software version 5.0 (San Diego, CA) was used for calculations.

Results

Tolerogenic effect of Bcl-2 inhibition by ABT-737

The effect of Bcl-2 inhibitors on BM engraftment and tolerance induction was first assessed by adding a short course of ABT-737 (50 mg/kg/day, from day −3 to day 12 after BMT) to a reduced conditioning protocol consisting of low-dose TBI (1.5 Gy) on the

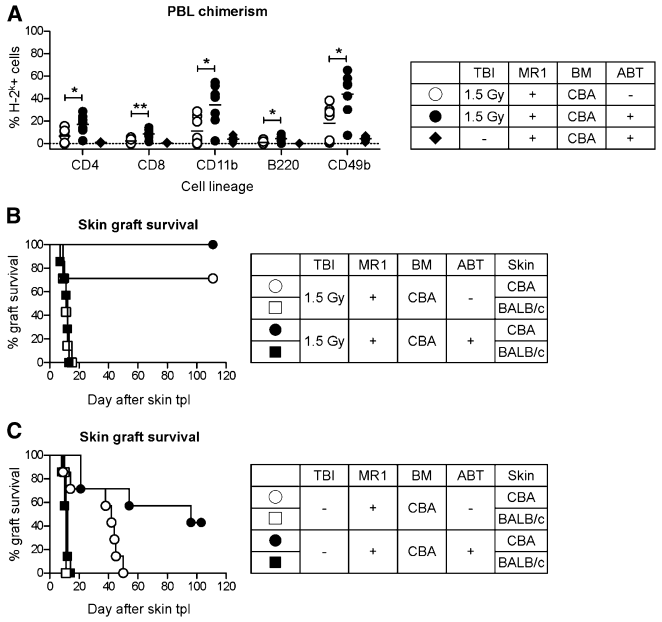

Figure 1. ABT-737 facilitates mixed chimerism induction. B6 recipients (H-2^b) were treated with a conditioning protocol including TBI (1.5 Gy), MR1 (2 mg), and 25 × 10^6 fully MHC-mismatched BM cells from CBA donors (H-2^k). Six weeks after BMT, skin transplantation from CBA and BALB/c (3rd party, H-2^d) donors was performed. (A) Adding a short course of ABT-737 to the conditioning regimen (ABT; 50 mg/kg/day, from day −3 to day 12 after BMT) resulted in a higher number of chimeric animals and significantly increased the percentage of donor-derived cells in different cell lineages in peripheral blood leukocytes (PBL; 10 weeks after BMT). CD11b for neutrophils; CD49b for natural killer cells; *$P < .05$; **$P < .01$. N = 7. (B) After skin transplantation all chimeric mice accepted donor grafts and promptly rejected 3rd-party grafts, demonstrating that donor-specific tolerance had been induced. N = 7-8 per group. (C) Using the same experimental protocol but without TBI, we obtained a pronounced donor-specific hyporesponsiveness, but tolerance was not achieved, as shown by the slow rejection of donor skin grafts over time. N = 7-8 per group. Representative results of 2 independent experiments are shown. tpl, transplantation.

day before BMT, a single injection of anti-CD154 (MR1, 2 mg) and 25 × 10^6 fully MHC-mismatched CBA BM cells to B6 recipients. ABT-737 increased the percentage of chimeric mice and induced a higher level of chimerism in all hematopoietic cell lineages (Figure 1A). All chimeric animals accepted donor-type skin grafts for more than 100 days and promptly rejected third-party grafts (BALB/c) (Figure 1B). In a second experiment, mice were treated with the same conditioning protocol including ABT-737, MR1, and BM cells, but without TBI. Although this protocol was not sufficient to allow BM engraftment (Figure 1A), skin transplantation (performed 6 weeks after BMT) revealed a marked and lasting donor-specific hyporesponsiveness in comparison with mice treated with MR1 and BM only (median survival time, 42 vs 96 days) (Figure 1C). Nevertheless, donor grafts were eventually rejected (graft survival 21, 21, 54, 96, >100, >100, and >100 days). Thus, inhibition of Bcl-2, Bcl-xL, and Bcl-w was not detrimental for survival and engraftment of donor-derived hematopoietic stem cells. Furthermore, ABT-737 in combination with CD154 blockade led to some degree of donor-specific hyporesponsiveness, but it was not sufficient to induce stable tolerance in an irradiation-free conditioning protocol.

We previously demonstrated that the proapoptotic effect of ABT-737 on naïve and activated lymphocytes can be potentiated in combination with CsA.[22,23] Therefore, we added a short course of low-dose CsA (10 mg/kg/day subcutaneously) to the mixed chimerism protocol introduced above (1.5 Gy TBI, MR1, 25 × 10^6 BM cells from CBA donors). In previous studies, treatment with CsA alone had an antitolerogenic effect and resulted in a reduced number of tolerant mice and lower chimerism levels (Figure 2A-B).[8,10,11] However, the combination of CsA with ABT-737 completely prevented this phenomenon and induced a high level of chimerism (Figure 2A; supplemental Figure 1 for kinetics of multilineage chimerism) and donor-specific tolerance in all recipients, as shown by the long-term survival of donor-type CBA skin grafts (Figure 2B). Importantly, the combination of ABT-737 with CsA not only prevented the antitolerogenic effect of calcineurin inhibitors, it resulted in an even higher chimerism level in comparison with ABT-737 alone, thereby reversing the antitolerogenic effect of CsA in a paradoxical synergism (Figure 2A). The mechanism underlying this clinically important finding was further investigated.

The critical role of the proapoptotic factor Bim in deletional tolerance

Because ABT-737 inhibits antiapoptotic Bcl-2 factors with high selectivity,[15] its tolerogenic effect is likely to result from a direct interaction with the regulation of the intrinsic apoptosis pathway in alloreactive lymphocytes. We hypothesized that ABT-737 might reverse the antitolerogenic effect of CsA by compensating a dysregulation of the intrinsic apoptosis pathway in activated CD8 T cells determined by signal 1 inhibition.[28] First, we aimed to identify an antiapoptotic factor of the Bcl-2 familiy, whose expression in donor-reactive T cells was reduced under MR1 alone, but not in combination with CsA. To monitor the regulation of Bcl-2 factors in a homogeneous population of alloreactive CD8 T cells, we took advantage of the BM3.3 transgenic mouse, which expresses on all CD8 T cells a transgenic TCR specific for the MHC class I molecule H-$2K^b$ and can be detected by the clonotypic antibody Ti98. BM3.3 splenocytes were stimulated with CBA (syngeneic) or B6 (allogeneic) splenocytes under the effect of CsA and MR1 in classical MLR experiments, and the expression of different Bcl-2 factors was measured in FACS. Among the most important antiapoptotic Bcl-2 factors, only the expression of Bcl-xL was reduced under the effect of MR1, but this process was not reversed in combination with CsA (Figure 3A). This

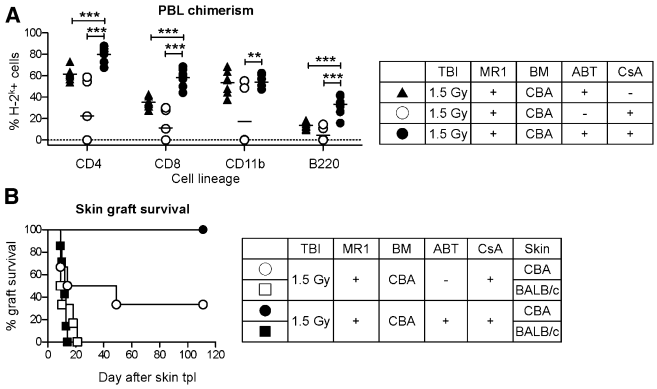

Figure 2. ABT-737 reverses the antitolerogenic effect of CsA. B6 recipients (H-2^b) were treated with a conditioning protocol including TBI (1.5 Gy), MR1 (2 mg), and 25 × 10^6 fully MHC-mismatched BM cells from CBA donors (H-2^k). Six weeks after BMT, skin transplantation from CBA and BALB/c (3rd party, H-2^d) donors was performed. (A) CsA (10 mg/kg/day, from day −3 to day 12 after BMT) had a deleterious impact on mixed chimerism induction, but this effect was reversed in combination with ABT-737 (ABT, 50 mg/kg/day, from day −3 to day 12 after BMT), as shown in the level of chimerism in different hematopoietic cell lineages in peripheral blood leukocytes (20 weeks after BMT). Notably, the chimerism level in the combination group was even higher than in the group treated with ABT-737 alone. $^{**}P < .01$; $^{***}P < .001$; N = 7-8. (B) Most recipients receiving CsA alone rejected CBA skin grafts, whereas all mice treated with a combination of CsA and ABT-737 accepted CBA and promptly rejected BALB/c skin grafts. N = 7-8/group.

finding was consistent with the previously reported critical role of Bcl-xL in this model,[13] but did not explain the effect of CsA and ABT-737. However, we found that CsA had a major impact on the regulation of Bim, a fundamental proapoptotic Bcl-2 factor in T cells. Alloantigen stimulation induced a transitory upregulation of Bim in CD8 T cells (Figure 3B). The initial upregulation phase was dependent on signal 1 (and was blocked by CsA), whereas the subsequent downregulation was influenced by costimulatory signals. As a result, the level of Bim remained low in cells activated in the presence of CsA, and—starting at day 3 after stimulation—was significantly higher in CD8 T cells treated with MR1 (Figure 3C). This correlated with the viability of polyclonally stimulated alloreactive CD8 T cells: costimulation blockade did not influence alloreactive CD8 T cell viability during the first 2 days of culture (not shown), but a progressive loss of viability in alloreactive CD8 T cells devoid of costimulation was registered at days 3 and 4 after stimulation. This process was completely dependent on Bim, as shown in experiments using Bim$^{-/-}$ CD8 T cells under the same experimental conditions (Figure 3D). Notably, the dysregulation of Bim under the effect of CsA was not influenced by MR1 (Figure 3C).

The critical role of Bim for deletional tolerance during mixed chimerism induction was confirmed in vivo by applying an established conditioning protocol (TBI 3 Gy, MR1) to Bim$^{-/-}$ recipients. In comparison with wild-type (WT) animals, Bim$^{-/-}$ mice displayed a marked resistance to mixed chimerism induction (Figure 3E). This was reflected in skin graft survival: in Bim$^{-/-}$ mice the majority of donor-type CBA skin grafts were rejected within 50 days in comparison with indefinite survival in WT mice (Figure 3F). Notably, ABT-737 failed to promote tolerance when added to the same conditioning protocol in Bim$^{-/-}$ recipients (Figure 3F-G). These data are consistent with the involvement of Bcl-xL and Bim in anti-CD154-dependent deletional tolerance, support the thesis that the antitolerogenic effect of CsA is related to a dysregulation of Bim, and indicate that ABT-737 promotes deletional tolerance and reverses the antitolerogenic effect of CsA by enhancing the function of Bim. This novel mechanism for inducing tolerance was explored to aim for a myelosuppression-free tolerance-induction protocol.

Irradiation- and myelosuppression-free tolerance induction by targeting the apoptosis pathway

WT B6 mice were treated with ABT-737 (50 mg/kg/day) and low-dose CsA (10 mg/kg/day) for 2 weeks starting at day −3 with respect to BMT, and an additional dose of both drugs was administered on day −2 in order to achieve more pronounced peripheral lymphocyte depletion. Subsequently, MR1 was injected 6-12 hours before 25 × 10^6 BM cells from fully MHC-mismatched CBA donors. This protocol led to multilineage mixed chimerism in 29 out of 33 mice in 5 independent experiments. Similar to previous attempts of mixed chimerism induction without myelosuppressive conditioning,[29] the level of donor chimerism was rather low in comparison with TBI-based protocols (Figure 4A-B). However, chimerism remained stable over time (supplemental Figure 2): 33 weeks after BMT, we measured a 4.4% of donor-derived CD4, 2.7% CD8, and 15.6% CD11b in the spleen. Interestingly, the B-cell compartment did not show any macrochimerism, either in the spleen or in peripheral blood at any time point after BMT. Donor-type CBA skin grafts were accepted indefinitely with a maximal observation time of 269 days, whereas all third-party grafts (BALB/c) were promptly rejected, therefore demonstrating donor-specific tolerance (Figure 4C). Notably, the additional dose of ABT-737 on day −2 was not sufficient to induce mixed chimerism without CsA (supplemental Figure 3)

Although the effect of CTLA4Ig on the regulation of Bcl-2 factors in vitro was promising (supplemental Figure 4), the success of this protocol was dependent on inhibition of the CD40/CD154 signaling, because the same conditioning regime failed to induce mixed chimerism and tolerance, when MR1 was replaced by CTLA4Ig (0.5 mg on day 2 after BMT; Figure 4C). The mechanisms of tolerance induction and maintenance in mice treated with this protocol were further investigated.

Robust peripheral and central deletional tolerance

First, tolerization of preexisting peripheral alloreactive T cells was assessed using the transgenic BM3.3 system described above. Before starting the tolerance induction protocol, 20 × 10^6 BM3.3 splenocytes were transferred into syngeneic CBA recipients, which allowed monitoring alloreactive BM3.3 CD8 T cells in blood over time using the clonotypic antibody Ti98. In mice treated with the full conditioning protocol including ABT-737 (50 mg/kg/day), low-dose CsA (10 mg/kg/day), MR1, and 25 × 10^6 B6 BM cells, donor-reactive CD8 T cells completely disappeared from the peripheral T-cell repertoire in the first 2 weeks and did not recover after resolution of lymphopenia (Figure 5A). In contrast, in mice treated with the same pharmacological conditioning regimen, but without BMT,

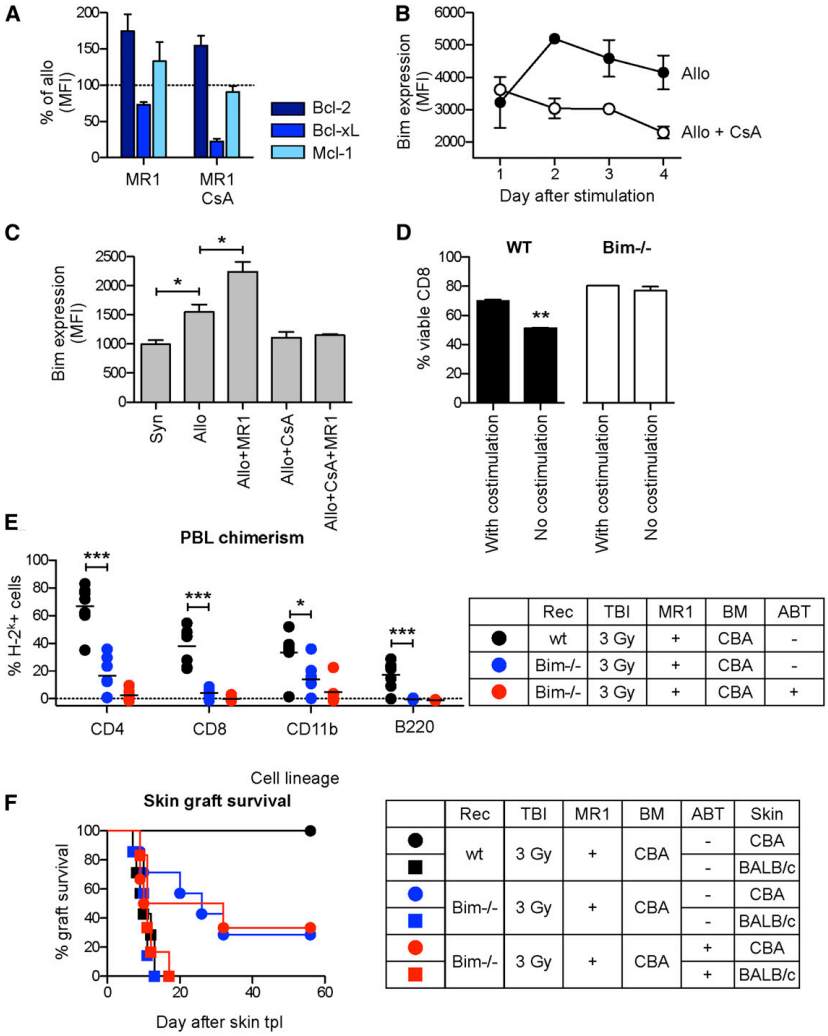

Figure 3. Bim is required for induction of mixed chimerism. BM3.3 splenocytes were stimulated in vitro with CD8-depleted B6 (allo) or CBA (syn) splenocytes. The expression of different Bcl-2 factors in transgenic alloreactive BM3.3 CD8 T cells was monitored by FACS. (A) In comparison with allostimulated cells without additional pharmacological treatment, after 4 days of MLR, cells exposed to MR1 expressed higher levels of Bcl-2 and Mcl-1 and lower levels of Bcl-xL. The expression of these antiapoptotic factors was not influenced by an additional treatment with CsA. Percentages of mean fluorescence intensity (MFI) values in comparison with allostimulated cells without pharmacological treatment are shown. (B) Allostimulation induced a transient upregulation of Bim, with a peak after 2 days of culture. CsA inhibited the initial upregulation of Bim, and MR1 prevented its downregulation in the late activation phase. (C) As a result, after 4 days the level of Bim was low in cells stimulated in the presence of CsA and high with MR1. Statistical comparison with syn: $^{*}P < .01$. (D) The relevance of these processes on CD8 T-cell viability after polyclonal stimulation was assessed culturing WT and Bim$^{-/-}$ splenocytes in the presence of anti-CD3 with anti-CD28 antibodies or without anti-CD28 and MR1. After 4 days, absence of costimulation reduced the viability of WT CD8 T cells, but the same phenomenon was not observed using Bim$^{-/-}$ cells, suggesting that the downregulation of Bim (C) was important for the viability of activated T cells. $^{**}P < .01$. (E) In vivo, a standard conditioning protocol (3 Gy TBI, MR1, 25 $\times$ 10^6 CBA BM cells) induced mixed chimerism in all WT B6 mice, but was not successful in the majority of Bim$^{-/-}$ mice, as shown by the levels of chimerism 15 weeks after BMT and (F) by the rejection of CBA skin grafts. Similar results were obtained if ABT-737 was added to the same conditioning protocol (E-F). Statistical comparison WT vs Bim$^{-/-}$: $^{*}P < .05$; $^{***}P < .001$; N = 6-7 per group.

Ti98+ cells were markedly reduced, but still detectable in peripheral blood. The complete deletion of Ti98+ cells in mice exposed to the full protocol was confirmed by FACS analysis of the spleen after rechallenging the recipient mice with B6 splenocytes (10^6 cells IV) (Figure 5B-C). Thus, exposure to donor-derived BM cells under the effect of MR1 and ABT-737 induced a complete peripheral deletion

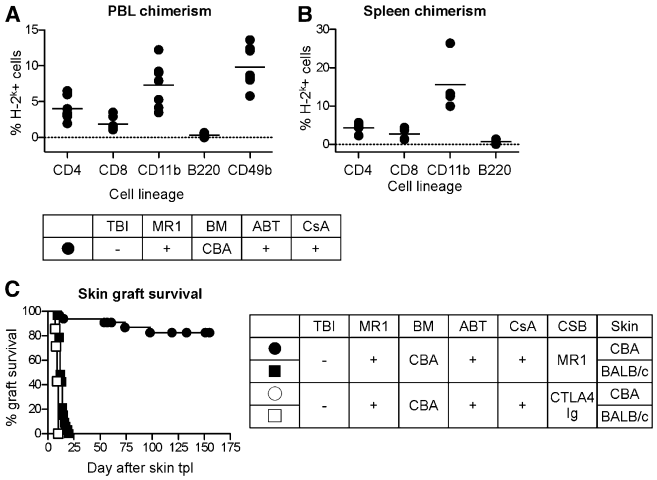

Figure 4. Myelosuppression-free tolerance induction with ABT-737 and CsA. B6 recipients (H-2^b) were treated with an irradiation-free conditioning protocol including ABT-737 (50 mg/kg/day), CsA (10 mg/kg/day) from day −3 to day 12, costimulation blockade (CSB) with MR1 (2 mg on day −1), and 25 × 10^6 fully MHC-mismatched BM cells from CBA donors (H-2^k). Four to 6 weeks after BMT, skin transplantation from CBA and BALB/c (3rd party, H-2^d) donors was performed. (A-B) A significant myeloid (CD11b+) and T-cell chimerism, but no chimerism in the B-cell compartment, was measured in peripheral blood (FACS at week 10 after BMT shown in panel A) and in the spleen (FACS at 33 weeks after BMT shown in panel B). N = 7 per group. Representative results of 5 independent experiments are shown. (C) Donor skin grafts were indefinitely accepted by 29 out of 33 mice in 5 independent experiments, whereas 3rd-party grafts (BALB/c) were promptly rejected. Combined data of 5 independent experiments are shown. N = 33. The same conditioning protocol failed to induce tolerance, when MR1 was replaced by CTLA4Ig (0.5 mg on day 2).

of alloreactive T cells during the mixed chimerism induction phase, and this was not impaired by CsA.

Second, the mechanism of long-term maintenance of tolerance was investigated in mice receiving our irradiation-free protocol. Mixed chimeras maintain donor-specific tolerance through central deletion of donor-reactive T cells, and the presence of donor-derived antigen-presenting cells in the thymus is critical in this setting.[30] Using the above-mentioned irradiation-free protocol, we consistently detected about 5% of donor-derived antigen presenting cells (CD11c+) in the thymus as demonstrated by thymic FACS analysis 33 weeks after BMT (Figure 6A), a finding consistent with central clonal deletion. The presence of a sustained systemic tolerance was further confirmed by different immunological tests: donor-specific B-cell tolerance was demonstrated by the absence of donor-specific IgG alloantibodies, whereas 3rd party–reactive IgG was readily detectable several weeks after skin grafting (Figure 6B). A lack of donor-specific T-cell responses was measured in classical MLR experiments (Figure 6C), confirming at a functional level a complete deletion of donor-reactive T cells from the peripheral repertoire. Finally, 58 days after the first skin transplantation, tolerant mice were rechallenged with a second skin graft from donor and 3rd-party control. All mice accepted the secondary CBA graft and rejected the BALB/c graft (Figure 6D-E). Taken together, a 2-week conditioning protocol with ABT-737 and CsA in combination with MR1 induced mixed chimerism and stable systemic T- and B-cell tolerance without myelosuppressive treatment and with moderate doses of BM cells.

Discussion

In this study we explored the intrinsic apoptosis pathway as a novel pharmacological target to induce mixed chimerism and allograft tolerance. We observed a critical role of the proapoptotic Bcl-2 family member Bim for deletional tolerance using costimulation blockade, and we therapeutically exploited this finding using the BH3-mimetic ABT-737 to establish a new irradiation- and myelosuppression-free protocol to induce mixed chimerism with a moderate dose of BM cells.

The tolerization of the peripheral T-cell compartment in mixed chimerism induction protocols based on costimulation blockade depends on the complex regulation of apoptosis in T cells after antigen recognition.[13] Focusing on the intrinsic pathway, the initial upregulation of Bim is counteracted by a simultaneous regulation of antiapoptotic factors and is required for T-cell activation.[28,31] Three to 4 days after antigen-recognition stimuli by costimulatory

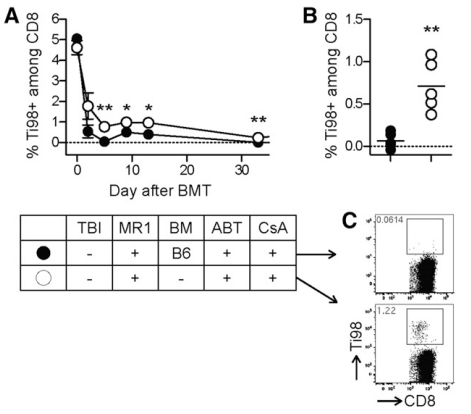

Figure 5. Peripheral deletion of alloreactive CD8 T cells. To monitor a well-defined population of alloreactive CD8 T cells during tolerance induction with ABT-737 and CsA, we adoptively transferred CBA recipients (H-2^k) with BM3.3 splenocytes (transgenic TCR specific for H-2K^b on all CD8 T cells) and subsequently treated with our irradiation-free conditioning protocol including ABT-737 (50 mg/kg/day), CsA (10 mg/kg/day) from day −3 to day 12, MR1 (2 mg), and 25 × 10^6 fully BM cells from B6 donors (H-2^b). In a control group, BM cells were not administered (conditioning only). (A) Transgenic donor-reactive BM3.3 CD8 T cells (Ti98+) were reduced in both groups, but only in mice receiving the full protocol was a complete deletion achieved. Statistical comparison of the 2 groups: *$P < .05$; **$P < .01$; N = 5. (B-C) After rechallenging with donor antigens (10^6 B6 splenocytes IV at day 37 after BMT), Ti98+ cells were readily detectable in the conditioning-only group, but had completely disappeared from the peripheral T-cell repertoire after exposure to the full protocol. **$P < .01$; N = 5 per group.

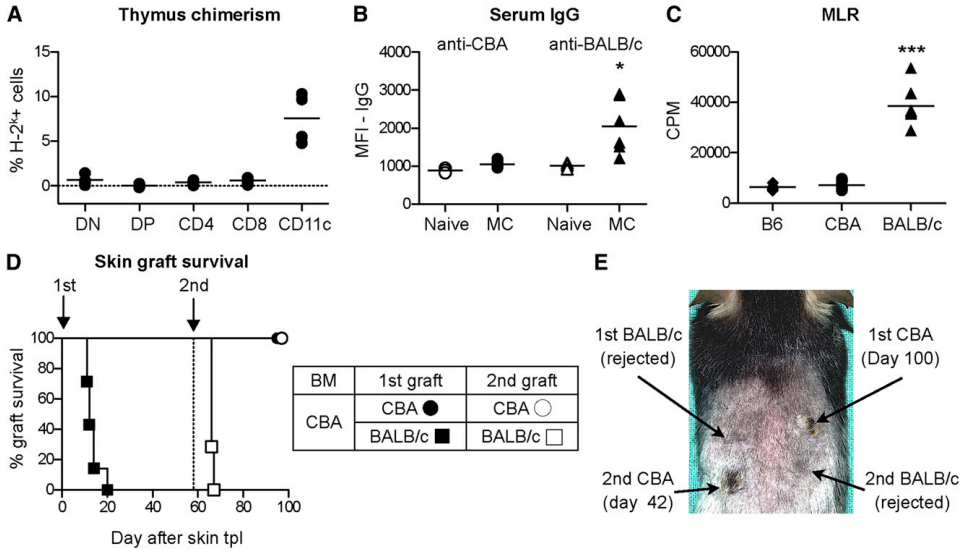

Figure 6. Robust, systemic tolerance after mixed chimerism induction with ABT-737 and CsA. B6 recipients were treated with an irradiation-free conditioning protocol including ABT-737 (50 mg/kg/day), CsA (10 mg/kg/day) from day −3 to day 12, MR1 (2 mg), and 25 × 10^6 BM cells from CBA donors. Six weeks after BMT, skin transplantation from CBA and BALB/c donors was performed. (A) FACS analysis 33 weeks after BMT revealed a significant percentage of donor-derived antigen-presenting cells (CD11c+) in the thymus, but not in T cells and in T-cell precursors (DN, double negative; DP, double positive). (B) Serum samples were collected 60 days after skin transplantation and analyzed by indirect FACS using CBA and BALB/c cells: a complete absence of CBA-reactive IgG and a normal seroconversion toward BALB/c were measured. MFI, mean fluorescence intensity. Statistical comparison between mixed chimeras (MC) and a group of naïve mice is shown. *$P < .05$; N = 5. (C) Thirty-three weeks after BMT, recipient mice were killed and their splenocytes stimulated in vitro with irradiated B6 (syn), CBA (BM donor), and BALB/c (3rd party) splenocytes in a classical MLR. T-cell proliferation analysis, measured by ^{3}H-thymidin incorporation, revealed a complete lack of T-cell reactivity against CBA and a normal response toward BALB/c. CPM, counts per minute; ***$P < .001$; N = 5 per group. (D) A group of mice received a second skin graft 58 days after initial transplantation. All BALB/c grafts were rejected within 10 days, whereas CBA grafts were accepted for more than 40 days without signs of rejection. N = 7 per group. (E) Photograph of a representative example at day 100 after first transplantation.

molecules and interleukins are required to maintain cell survival by a downregulation of Bim and an upregulation of Bcl-xL.[32,33] In the absence of an adequate antiapoptotic signal through costimulation, Bim prevails and activated T cells die by apoptosis.

Bcl-2 inhibitors offer the opportunity to selectively interact with these mechanisms. In our model, ABT-737 promoted peripheral T-cell tolerization by at least 2 complementary effects. First, it reduced the precursor frequency by a peripheral depletion of lymphocytes. Second, it led to a more efficient clonal deletion of alloantigen-reactive T cells by directly boosting the critical role of Bim in combination with anti-CD154. Additional effects of Bcl-2 inhibitors on regulatory T cells cannot be excluded. The beneficial effect of the combination of ABT-737 with CsA is multifactorial. CsA potentiated the proapoptotic effect of ABT-737 on lymphocytes and blocked the upregulation of A1, thereby preventing resistance to ABT-737 in T cells after antigen recognition.[22,23] On the other hand, ABT-737 reversed the antitolerogenic effect of CsA by compensating the missing upregulation of Bim after allostimulation under calcineurin blockade (Figure 3). As a result, the combination of CsA and ABT-737 resulted in a paradoxical synergistic effect. This finding is of critical clinical relevance: because graft lost because of acute rejection cannot be ethically accepted in the modern transplantation era, a clinically applicable tolerance induction protocol has to foresee a combination of initial standard immunosuppressive and tolerance-induction regimes in a preventive or therapeutic setting, ideally without the a priori exclusion of calcineurin inhibitors.[34]

A short induction phase with ABT-737, CsA, and MR1 induced a complete deletion of peripheral donor-reactive T cells and allowed the engraftment of a sufficient number of donor-derived stem cells to maintain a durable mixed hematopoietic chimerism. Notably, because hematopoietic stem cells express high levels of Mcl-1,[35] ABT-737 does not induce apoptosis in this cell population and is not expected to "create space" in stem cell niches, a factor that was originally thought to be required for BM engraftment.[36,37] A significant level of chimerism without myelosuppressive conditioning was previously achieved using megadoses of BM, but has never been reported using clinically applicable BM doses.[29] Because our conditioning protocol was sufficient to induce a stable level of chimerism >5% with moderate doses of BM, we speculate that ABT-737 and CsA might promote BM engraftment by influencing the complex interaction of immunological, stromal, and stem cells in the stem cell niche[38] or by promoting the physiological niche recycling.[39] These aspects might assume a general relevance for BM transplantation and the underlying mechanisms merit further investigation.

The sustained detection of donor-derived granulocytes over more than 8 months clearly indicated that engraftment of donor stem cells or very early progenitor cells had occurred. However, the level of hematopoietic chimerism was not uniform among different cell lineages with a complete absence of donor-derived B cells and higher levels of granulocytes. This could be explained by the engraftment of myeloid-biased hematopoietic stem cells.[40,41] The donor-derived

hematopoiesis was sufficient to maintain a pool of donor-derived antigen-presenting cells in the thymus to establish central deletional tolerance and—in a clinical perspective—may be favorable, because it reduces the risk of graft-vs-host disease.

The tolerance induction protocol described here provides a solution to several problems currently precluding a broad clinical application of the mixed chimerism approach. The general toxicity of the conditioning regimen is low in comparison with protocols including myelosuppressive drugs or TBI.[4] In phase I clinical trials, ABT-263 displayed a favorable toxicity profile, and its application for a short conditioning therapy seems to be adequate also for nonmalignant conditions.[21] Depleting antibodies—notably more efficient in mice than in humans[42]—are not required, but a blockade of the CD40/CD154 pathway was critical in our model. The recent report of antibodies blocking CD40 may represent an ideal solution for blocking this pathway without the thromboembolic side effect reported in primates after exposure to anti-CD154.[43-46] Importantly, in contrast to previous reports of mixed chimerism induction without myelosuppression,[29,47] our conditioning protocol was successful using a clinically relevant dose of BM cells and without additional cell-based therapy.

In summary, we established a novel and reliable approach to induce mixed chimerism and allograft tolerance by pharmacological modulation of the intrinsic apoptosis pathway. This approach allowed induction of mixed chimerism using a nontoxic, nonmyelosuppressive conditioning protocol with potential clinical applicability.

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Authorship

Contribution: P.E.C. and T.F. designed and performed experiments and wrote the paper; J.C., S.S.G., and A.K.K. performed experiments; A.B. and T.W. provided important scientific input; P.D.B., A.G., and T.W. provided important reagents; and R.P.W. supervised the project.

Conflict-of-interest disclosure: P.D.B. is an employee of Abbott, which developed and provided ABT-737; however, no financial sponsoring was received for this study. The remaining authors declare no competing financial interests.

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